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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of: Jackson et al.

Group Art Unit: 1647

Serial No.: 08/942,596

Examiner: S. Turner

Filed: October 2, 1997

Attorney Docket No.: 7969-062

For: CHLAMYDIA PROTEIN, GENE SEQUENCE AND USES THEREOF

Second Declaration of Dr. W. James Jackson Under C.F.R. § 1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

SIR:

I, W. James Jackson, declare and state as follows:

1. I am a co-inventor of the above-identified application which I have read and understand.

2. The following experiments were conducted either personally by me or under my guidance and supervision. Results obtained are shown in the Tables below. The results obtained clearly demonstrate that isolated *Chlamydia* HMW protein in combination with an adjuvant induced a protective immune response against *Chlamydia* infection.

3. *Chlamydia* HMW protein of the present invention comprising an amino acid sequence which is about 99.4% identical to SEQ ID NO.: 2 was expressed recombinantly in *E.coli* (rHMW protein) and obtained in isolated form as taught in the specification of the above-identified application.

4A. METHODS

The Tuffrey murine infertility model was employed to evaluate the efficacy of rHMW protein to protect animals against *Chlamydia trachomatis*-induced salpingitis and infertility. Three groups of 17 female C3H HeOuJ mice (~6 weeks of age, Jackson Labs) were employed for this evaluation. The test group of 17 animals was immunized at weeks 0, 2, and 3 by intranasal administration of ~20 µl of a vaccine formulation containing approximately 10-12 µg of gel-purified rHMW protein and ~5 µg mLT (SmithKline Beecham) as adjuvant. Prior to immunization mice were sedated using an anesthesia cocktail consisting of 16% Ketaject and 16% Xylaject in 68% pyrogen-free PBS (100 µl i.p./animal). Sedated animals were placed on their backs and using a standard laboratory pipette administered the vaccine formulation; ~10 µl of the vaccine solution per nostril with a 5-10 minute wait period between applications.

Two groups of 17 female mice (per test group) were immunized similarly but with a preparation containing only 5 µg mLT (*i.e.* adjuvant only, no antigen). One of these groups was subsequently challenged with *C. trachomatis* (sham immunized, infected) and served as the negative fertility control while the other group was not challenged (sham immunized, sham infected) and served as the positive fertility control.

At week 4, all animals were administered a single i.p. dose of progesterone (2.5 mg in pyrogen-free PBS, Depo-Provera, Upjohn) to stabilize the uterine epithelium. At week 5, animals immunized with rHMW protein and animals in the negative control group were infected by bilateral intrauterine inoculation with ~$5X10^5$ inclusion forming units (IFU) of *C. trachomatis* NI1 (serovar F) in 100 µl of sucrose phosphate glutamate buffer (SPG). To mimic the manipulations to the reproductive tract experienced by the other two groups, animals in the positive control were bilaterally inoculated with 100 µl of a McCoy cell extract that contained no *C. trachomatis*. At week 7, 5-7 animals from each group were sacrificed by CO_2 asphyxiation and the complete genital tract (both upper and lower reproductive tracts) removed for histopathological analysis. At week 9, the remaining females from each group were caged with 8 - 10 week old male C3H mice for a 2 month breeding period to assess fertility (1 male for every 2 females per cage with weekly rotation of the males within each group, animals from different experimental groups were not mixed). Palpation and periodic weighing were used to determine when animals in each pair became pregnant. The parameters used to estimate group fertility were: F, the number of mice which littered at least

once during the mating period divided by the total number of mice in that study group; M, the number of newborn mice (born dead or alive) divided by the number of litters produced in that group during the mating period; and N, the number of newborn mice (born dead or alive) divided by the total number of mice in that group.

HISTOPATHOLOGY

Genital tracts were treated for ≥ 24 hrs in Bouin's fixative, progressively dehydrated in 50%, 70%, and 100% methanol, soaked in toluol, and either paraffin embedded or directly embedded in OCT compound (Tissue-TEK, Miles) and subsequently snap frozen in liquid nitrogen. Tissue sections (~6 μm) were stained with hematoxylin and eosin (after deparaffinization of the Bouin fixed samples). Inflammatory changes in the oviducts and ovaries were graded as follows: 0, no apparent inflammatory reaction; 1, a few mononuclear cells infiltrating the periovarial space or the submucosa of the oviduct; 2, same as 1 but to a greater extent; 3, same as 2 but with a thickened oviductal submucosa and the presence of inflammatory cells in the oviductal lumen; 4, same as 3 but to a greater extent. Inflammation in the cervix/vagina was scored based on the level of the intraepithelial infiltrate observed.

DETERMINATION OF rHMW protein-SPECIFIC HUMORAL RESPONSES

Blood samples were collected periodically during the immunization and challenge phases by retroorbital bleeding and serum prepared by centrifugation. Vaginal secretions were collected by repeated injection of 50 μl of sterile PBS into the vagina with a standard laboratory pipettor and immediately withdrawing the solution. Two-to-three injection/withdrawal cycles were performed.

Quantitation of antibody (Ab) responses by ELISA were performed as described in Section 8, Example 11 of the specification of the application. Microwell ELISA plates (Maxisorb, NUNC) for determining Ab levels were coated overnight at 4°C with ~0.5-1.0 μg of gel-purified rHMW protein per well in 10mM carbonate/bicarbonate buffer (pH 9.6), washed with PBS containing 0.1% Tween-20 (washing buffer) and blocked for ~1hr at 37°C with a PBS solution containing 3% BSA. For the determination of antigen-specific serum IgG levels, test sera were serially diluted in washing buffer containing 0.5% BSA and aliquots (100 μl) incubated in the antigen-coated wells for ~2hr at 37°C. The plates were then washed and incubated for ~1hr at 37°C with a horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG second antibody (Sigma). A HRP-conjugated goat anti-mouse IgA secondary antibody was used to detect the presence of rHMW protein-specific IgA in vaginal secretions.

After incubation with the appropriate secondary Ab, the plates were washed and incubated for ~20-30 minutes at room temperature with TMB substrate (Sigma). Reactions were stopped by the addition of 2M H_2SO_4 and the absorbance determined at 450 nm on a Molecular Devices SpectroMax microplate reader. Titers were determined as the reciprocal of the sample dilution corresponding to an optical density of 1.0 at 450 nm.

DETERMINATION OF rHMW protein-SPECIFIC CELLULAR RESPONSES

Groups of 6 female C3H HeOuJ mice (Jackson Labs) were sedated and immunized at weeks 0, 2, and 3 by intranasal administration with the rHMW protein + mLT vaccine as described above. At weeks 4 and 5 immediately prior to progesterone treatment and intrauterine challenge, respectively, 3 animals from each group were sacrificed by CO_2 asphyxiation and spleens aseptically removed and single cell suspensions prepared using conventional methodologies. Spleen cells from immunized animals were analyzed separately. For both the positive control group (sham immunized and sham infected) and the negative control group (sham immunized, infected) spleen cells were pooled and tested for restimulation.

For the measurement of spleen cell proliferation, spleens were ground (5 to 10 rounds) in 5ml of RPMI 1640 Glutamax I supplemented with 10% fetal calf serum, 25 mM HEPES, 50 U/ml penicillin, 50 µg/ml streptomycin, 1 mm sodium pyruvate, nonessential amino acids, and 50 µM 2-mercaptoethanol (Gibco-BRL). Live cells were counted by Trypan Blue staining and diluted in the same media to reach a density of 1.0 - 2.0X10^6 cells/ml (Falcon 2063 polypropylene tubes). Triplicate cultures were set-up in round bottom 96-well culture plates (Nunclon, Nunc) using ~5X10^5 responder cells per well in 200 µl of media Cells were stimulated with either 1.0µg/ml of rHMW protein (antigen-specific proliferation) or with 4 µg/ml concanavalin A (Boerhinger Mannheim) as a positive stimulation control; unrestimulated cell cultures were used as a negative control of cellular activation. After 72-96 hours of incubation at 37°C in 5% CO_2 cells were pulsed labelled for ~18hrs with 1.0 µCi ^{3}H-thymidine (Amersham) per well. Pulsed cells were harvested onto glass-fiber sheets using a Tomtec Cell Harvester (Mk III) and counted for beta-emission in a 3-channel Wallac 1450 Trilux Liquid Scintillation Counter. The stimulation index (SI) for a sample (individual or pooled) was defined as the mean of the antigen or ConA-stimulated T-cell uptake of ^{3}H-thymidine for triplicate wells divided by the mean of the unstimulated

uptake for triplicate wells. SIs for both antigen-specific (rHMW protein-specific) and ConA-specific proliferation were determined.

4B. RESULTS

EFFECT ON MOUSE FERTILITY AFTER A HETEROTYPIC CHALLENGE

Evidence that mucosal immunization with rHMW protein combined with mLT affords protection against infertility caused by a human clinical isolate of *C. trachomatis* (strain NI1, serovar F) is shown in Table 1. Animals immunized with the rHMW protein displayed a significantly higher fertility rate (70%, i.e. number of fertile females in group/total number of animals in the group) than animals in the negative control group (30%, sham immunized and infected). Similarly, the rHMW protein immunized group produced more offspring and exhibited a group fecundity greater than those observed in the negative control group (51 vs 24 offspring and 5.1 ± 4.7 vs 2.4 ± 4.6 fecundity scores, respectively). As a group, animals immunized with the rHMW protein vaccine displayed a comparable fertility rate, total number of offspring, and a fecundity score to those observed in the sham infected positive control group (80% fertility rate, 56 total offspring, 4.9 ± 2.7 fecundity).

The protection against *C. trachomatis*-induced infertility obtained in this experiment also demonstrates the utility of the rHMW protein to afford cross-biovar and cross-serovar protection against *C. trachomatis* disease. The recombinant HMW protein antigen employed in this experiment was cloned from a strain belonging to the *C. trachomatis* lymphogranuloma venereum (LGV) group (strain L2) which causes systemic as well as more localized mucosal infections of the eye and genital tract. The *C. trachomatis* challenge organism used in these experiments, strain NI1 is an F serovar organism that belongs to the trachoma biovar which causes numerous urogenital tract infections.

Table 1. Fertility Assessments Observed After ~2 Breeding Cycles

Group	Number of Animals per Group	Percent Fertile Animals	Number of Off-spring	Group Fecundity[1] (Mean + SD)
rHMW protein-Immunized	10	70 p = 0.089[2]	51	5.10 ± 4.68 p = 0.105[3]
Sham Immunized Sham Infected (Positive Control)	10	80 p = 0.035	56	4.90 ± 2.70 p = 0.078
Sham Immunized Infected (Negative Control)	10	30	24	2.40 ± 4.61

1 Mean number of pups per group

2 Fisher's exact test, one-sided, 95% confidence interval p-values are given relative to the negative control

3 Student's t-test, unpaired, Gausian distribution, 95% confidence interval p-values are given relative to the negative control

EFFECT ON CELLULAR IMMUNE RESPONSE

The rHMW protein-specific activation of the cellular immune system was demonstrated using a conventional spleen cell proliferation assay. When spleen cells were tested during week 4 (immediately prior to progesterone treatment) (Table 2) and week 5 (~7 days after hormone treatment but before intrauterine challenge) (Table 3), all samples collected from rHMW protein-immunized animals developed a strong antigen-specific proliferative immune response. The antigen-specific Stimulation Indexes (SIs) obtained prior to progesterone treatment from rHMW protein-immunized animals were equal to or greater than the SIs obtained via mitogenic stimulation with ConA (mean values for antigen and ConA stimulation obtained from 3 rHMW protein-immunized animals: 26.2 vs 18.4, respectively). Spleen cells obtained from either sham immunized animals or naive animals (i.e. animals that were not exposed to either the rHMW protein antigen or mLT) did not respond to in vitro restimulation with the rHMW protein material, thus establishing the specificity of the proliferative response observed in the immunized animals. Progesterone treatment did not affect the antigen-specific proliferative response observed in rHMW protein immunized animals. Antigen-specific SIs obtained with spleen cells obtained after hormone

treatment were greater than obtained via mitogenic stimulation (mean values for antigen and ConA stimulation obtained from 3 rHMW protein-immunized animals: 92.4 vs 37.8, respectively). Again samples collected from sham immunized or naive animals failed to demonstrate any antigen-specific proliferative response.

Table 2. rHMW protein-Specific Cell Proliferation Before Hormone Treatment

Group	Cell Proliferation (cpm) Untreated / ConA / rHMW protein	Stimulation Index (Treated cpm/ Untreated cpm) ConA / rHMW protein
rHMW protein Immunized Animal #1	1557 / 20739 / 65741	13.3 / 42.2
rHMW protein Immunized Animal #2	1508 / 26975 / 28361	17.9 / 18.8
rHMW protein Immunized Animal #3	1238 / 29991 / 23453	24.0 / 18.9
Sham-Immunized Animals (Pooled)	1687 / 30546 / 1292	18 / <1.0
Naive Animals (Pooled)	335 / 23886 / 838	71 / 2.5

Table 3. rHMW protein-Specific Cell Proliferation After Hormone Treatment

Group	Cell Proliferation (cpm) Untreated / ConA / rHMW protein	Stimulation Index (Treated cpm/ Untreated cpm) ConA / rHMW protein
rHMW protein Immunized Animal #1	767 / 15934 / 97458	20.8 / 127.0
rHMW protein Immunized Animal #2	546 / 17212 / 28172	31.5 / 51.6
rHMW protein Immunized Animal #3	297 / 18139 / 29300	61.1 / 98.6
Sham-Immunized Animal (Pooled)	273 / 18094 / 150	66.3 / <1.0
Naive Animals (Pooled)	345 / 16740 / 1341	48.5 / 3.9

EFFECT ON HUMORAL IMMUNE RESPONSE

To demonstrate that immunization with the full length rHMW protein produces a humoral immune response, IgG titers were measured by ELISA on sera collected at week 5 immediately prior to challenge (i.e. approximately 2 weeks after the third immunization). As shown in Table 4, immunization of C3H mice with three doses of ~10-12 μg rHMW protein produced detectable levels of anti-rHMW protein IgG in all animals. Vaginal secretions were also collected from these animals at the same time and tested for the presence of anti-rHMW protein mucosal IgA. Antigen-specific vaginal IgA was detected in three animals (Table 4).

Table 4. rHMW protein-Specific Humoral Response

rHMW protein Immunized Animal	Anti-rHMW protein Serum IgG ELISA Titer	Presence of Anti-rHMW protein Vaginal IgA
4.4	5,000	
4.5	6,000	
4.6	12,000	+
4.7	130	
4.8	100	
4.9	54,000	+
4.10	670	
4.11	100	
4.12	570	
4.13	100,000	+
4.14	4,500	
4.15	400	
4.16	1,600	
4.17	2,500	
4.18	700	
4.19	70,000	
4.20	500	
4.21	2,000	
4.22	18,000	
4.23	3,000	

5. In summary, the results presented above clearly demonstrate that immunization of experimental animals with isolated *Chlamydia* HMW protein and adjuvant induced a significantly higher fertility when challenged with *Chlamydia* in comparison to the non-immunized negative control group. In addition, the immunization resulted in a fertility rate

comparable to non-infected animals. Further, HMW-specific cellular and humoral immune responses were observed.

6. Based on the data presented above and on my expertise, it is my conclusion that one skilled in the art having the teaching of the present application and the data presented herein would understand and appreciate that the presently claimed isolated *Chlamydia* HMW protein, in combination with an adjuvant, is useful to induce a protective immune response against *Chlamydia*.

7. I have once again carefully reviewed the teaching of U.S. Patent No.: 5,725,863 to Daniels. I note that the only *Chlamydia* strain from which Daniels attempted to obtain any protein was *Chlamydia psittaci* strain DD-34. Hence, the Daniels Patent teaches nothing regarding proteins that might be isolated from another *Chlamydia*, including *C. trachomatis*, *C. pecorum* or *C. pneumoniae*. Moreover, based on my careful review and my expertise, it is my conclusion and opinion that nothing in the Daniels patent would have motivated any one skilled in the art to isolate any of the possible proteins from the mixture of proteins having molecular weights in the range of 40 to 140 kDa. In fact, since Daniels teaches that the proteins of that mixture did not have the ability to induce an immune response against *Chlamydia*, it is my opinion that this reference actually teaches away from isolation of any protein in such molecular weight range for use as a possible immunogen as a vaccine component.

I declare further that all statements made in this Declaration of my own knowledge are true and that all statements made on information and belief are believed to be true and further that these statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the specification or any patent issuing thereon.

Dated: 7/1/02

Dr. W. James Jackson

EXHIBIT B

#36
9/23/02

Express Mail No.: EL 500 575 975 US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of: Jackson et al.

Group Art Unit: 1647

Serial No.: 08/942,596

Examiner: S. Turner

Filed: October 2, 1997

Attorney Docket No.: 7969-062

For: CHLAMYDIA PROTEIN, GENE SEQUENCE AND USES THEREOF

Third Declaration of Dr. W. James Jackson Under C.F.R. § 1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

S I R:

I, W. James Jackson, declare and state as follows:

1. I am a co-inventor of the above-identified application which I have read and understand.

Considered
12-27-02
SC

2. The following experiments were conducted either personally by me or under my guidance and supervision. Results obtained are shown in attached Exhibit A1. The results obtained clearly demonstrate that isolated *Chlamydia* HMW protein, alone or in combination with an adjuvant, induced a protective immune response against *Chlamydia* genital tract (vaginal) infection in a relevant animal model.

3. *Chlamydia* HMW protein of the present invention comprising an amino acid sequence which is about 99.4% identical to SEQ ID NO.: 2 was expressed recombinantly in *E.coli* (rHMW protein) and obtained in isolated form as taught in the specification of the above-identified application.

3A. METHODS

Two Groups of six (6) female C3H HeOuJ mice were administered three nasal doses of a vaccine composed of recombinant HMW protein (10μg) alone or together with an adjuvant, i.e., mLT (5μg) in buffer (PBS) over the course of three weeks (days 0 – 14 – 21). Two groups of mice, i.e., two different control groups, were administered three nasal doses of either adjuvant alone (mLT) or buffer (PBS).

Approximately seven days after the last boost, animals were given a single dose of progesterone (2.5mg, i.p., Depo-Provera, Upjohn) to stabilize the uterine and vaginal epithelium. One week later, animals were challenged by instilling a ~20-25μl volume of a *C. trachomatis* serovar E lysate containing ~1 X 10^6 Inclusion Forming Units (IFU) directly into the vaginal vault. Periodically after challenge, polyester/dacron pediatric swabs were used to swab individual vaginas to recover infectious *C. trachomatis* elementary bodies (EBs). Infectious EBs were recovered from the swabs into SPG buffer, serially diluted, and appropriate dilutions used to infect monolayers of McCoy cells grown in standard 24-well microplates. Infected monolayers were incubated ~48hr at 37°C/5% CO_2 to allow the formation and growth of intracellular *C. trachomatis* inclusions. Inclusions were stained using a commercially available anti-*Chlamydia* LPS monoclonal antibody conjugated to FITC (PathFinder, Sanofi) and quantified by indirect fluoresence microscopy.

3B. RESULTS

The results obtained are presented in the graph attached as Exhibit A1. The level of residual *C. trachomatis* present in the vagina is expressed as inclusion forming units vs day post-challenge.

Also presented, for comparison, in Exhibit A1, are results obtained for another group of animals designated "previously infected". These non-immunized animals were infected with *C. trachomatis* about 30 days prior to challenge of all the experimental and control groups described above. These animals were also re-challenged with *C. trachomatis* at the same time as the experimental and control animals were challenged. The protocols for infection and challenge of the "previously infected" group were the same as for all the other groups.

4. As illustrated in Exhibit A1, at three days post-infection with live *Chlamydia*, vaginal clearance of *Chlamydia* was significantly better in animals immunized with isolated

Chlamydia HMW protein with adjuvant when compared to both animals immunized with adjuvant or PBS alone. As further illustrated in Exhibit A1, at seven days post-infection with live *Chlamydia*, vaginal clearance was significantly better in animals immunized with isolated *Chlamydia* HMW protein, with or without adjuvant, when compared to both animals immunized with adjuvant or PBS alone. The protection against infection with *Chlamydia* was equivalent to that induced by previous *Chlamydia* infection at post-seven days exposure.

5. Thus, the results presented in Exhibit A1 clearly demonstrate that immunization of experimental animals with the isolated *Chlamydia* HMW protein, with or without adjuvant, induced a protective immune response that was effective against genital tract (vaginal) infection by live *Chlamydia*. The results clearly demonstrate that the *Chlamydia* vaccine comprising isolated HMP protein, alone or with an adjuvant, protected the animals from infection with *Chlamydia*.

6. Attached, as Exhibit A2, is a copy of Rank et al., (Methods in Enzymology, Vol. 235: 83-93 (1994). As detailed at page 93 of Exhibit A2, the mouse model used in the studies described above is an art recognized model to study and assess *Chlamydia* infection. Hence, it is my considered opinion that those skilled in the art would understand the results discussed above to demonstrate efficacy of the novel vaccine compositions in an art recognized animal model.

7. Further I have read the comments in the Office Action dated July 16, 2002, at paragraph 7 on pages 3-4 with respect to Claims 57-60. I have also read and understand these claims to be directed to vaccine compositions containing an adjuvant and the novel isolated *Chlamydia* HMW protein described in the present application. The Office Action asserts that these vaccine claims are not supported by the application because the data presented in the application and in my earlier Declaration dated April 18, 2002 (Second Jackson Declaration) show that the "preparation fails to provide immunity such that the animals are resistant to infection". The Office Action states that a vaccine is recognized as a "preparation introduced into the body to produce immunity", and that "immunity" is defined as "exemption or freedom from something, resistance to or protection against a specified disease". The Office

Action then concludes that a vaccine requires that the "immunity" induced must be protective of the host against "infection" by the disease agent.

8. For a number of important reasons, this conclusion in the Office Action is in error.

Firstly, as detailed above in paragraphs 3-5, the results presented in attached Exhibit A1 clearly demonstrate that administration of a vaccine composition described in the application containing isolated *Chlamydia* HMW protein, alone or in combination with an adjuvant, induced a protective immune response against *Chlamydia* infection.

Secondly, the results presented in my Second Jackson Declaration would be understood by those skilled in the art to constitute evidence demonstrating that the immune response induced by the composition containing the novel isolated *Chlamydia* HMW protein of the application is indicative of an effective vaccine against *Chlamydia*. The results presented in the Second Jackson Declaration were obtained using the Tuffrey mouse infertility model. Attached as Exhibit A3 is a copy of Tuffrey et al., 1990, J. Repod. Fertility, entitled "Correlation of Infertility with Altered Tubal Morphology and Function in Mice with Salpingitis Induced by a Human Genital-tract Isolate of *Chlamydia trachomatis*" (Tuffrey). Tuffrey teaches the method employed in the experiments described in the Second Jackson Declaration to evaluate the ability of the claimed vaccine composition against Chlamydial infection. As clearly taught by Tuffrey, as the salginpitis in this mouse in this mouse model "closely resembles the human disease in its pathology and consequences for fertility", this model is "particularly relevant for research on Chlamydial vaccine development". Tuffrey Abstract at page 295; also page 304, last paragraph.

9. Based on the teaching of the present application, the results in the Second Jackson Declaration, the results described above and the evidence presented in the attached Exhibits, it is my considered opinion, that one skilled in the art would understand the data presented to demonstrate that the present compositions would be useful as a vaccine against *Chlamydia*.

I declare further that all statements made in this Declaration of my own knowledge are true and that all statements made on information and belief are believed to be true and further that these statements and the like so made are punishable by fine or imprisonment, or both,

under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the specification or any patent ~~issuing~~ thereon.

Dated: 8/29/02

Dr. W. James Jackson

EXHIBIT A1

Vaginal Clearance in HMWP Immunized Mice

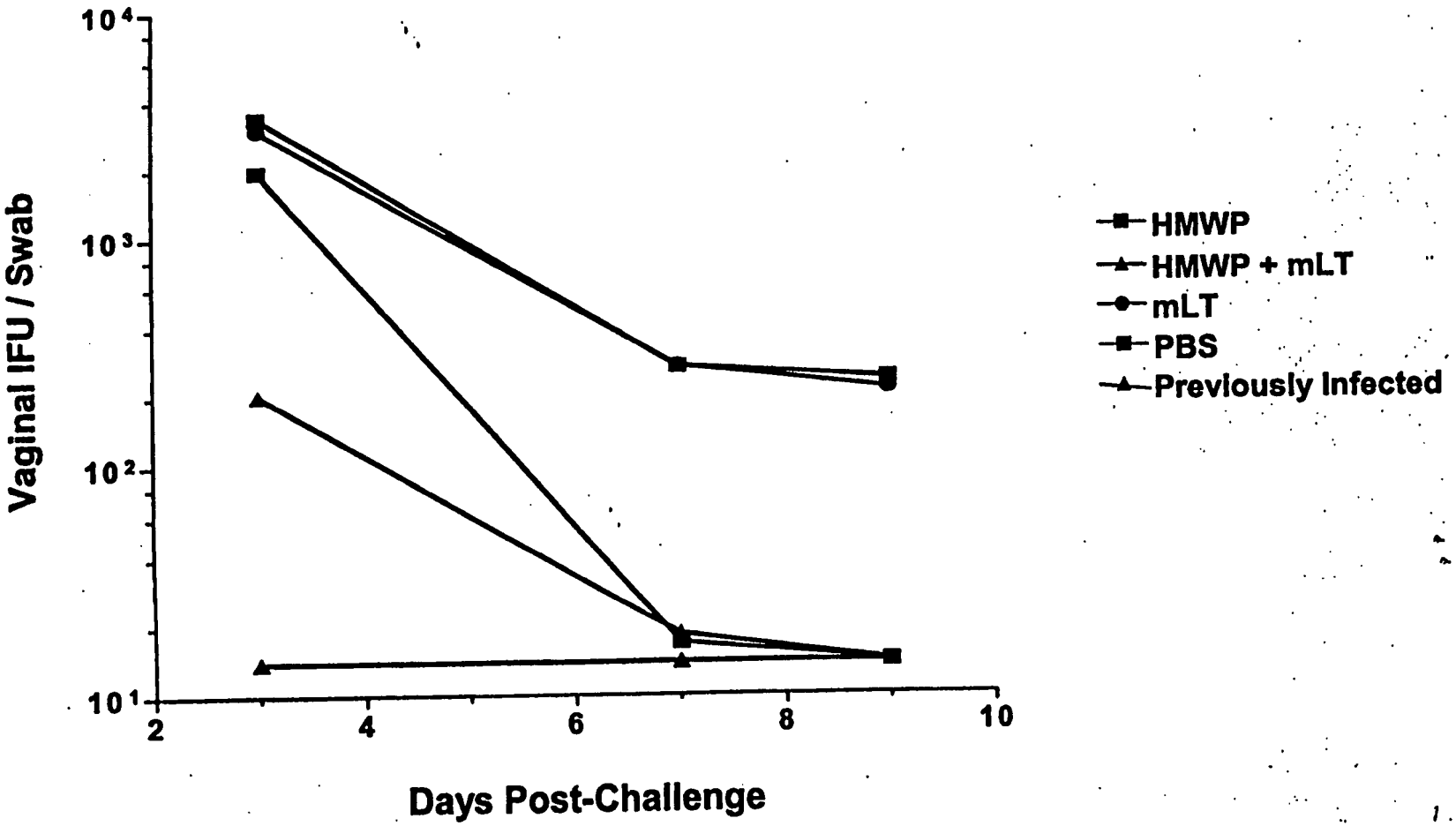

EXHIBIT A2

Methods in Enzymology

Volume 235

Bacterial Pathogenesis

Part A

Identification and Regulation of Virulence Factors

EDITED BY

Virginia L. Clark
Patrik M. Bavoil

SCHOOL OF MEDICINE AND DENTISTRY
UNIVERSITY OF ROCHESTER
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1 1/2-inch 22-gauge needle into a syringe containing a solution of trisodium citrate (44 g/liter), citric acid (16 g/liter), and glucose (50 g/liter) (0.1 ml per milliliter of blood). Approximately 4 ml of blood may be safely collected from a 500-g animal. The PBM are isolated on a Histopaque gradient (specific weight 1.077 g/ml; Sigma), washed, and counted.[30] Usually, about $2–6 \times 10^6$ cells can be obtained, which is sufficient for assays of multiple antigens or mitogens. If more cells are required or an increased purity of a T cell population is needed, spleens may be used. The T cells can be enriched by filtration of spleen cells over a nylon wool column using standard procedures.[31] For the response to GPIC, 1 μg per well (96-well microculture plate) of purified elementary bodies has been found to produce an optimal response.

[30] R. G. Rank, L. S. F. Soderberg, M. M. Sanders, and B. E. Batteiger, *Infect. Immun.* **57**, 706 (1989).
[31] J. U. Igietseme and R. G. Rank, *Infect. Immun.* **59**, 1346 (1991).

[7] Animal Models for Urogenital Infections

By ROGER G. RANK

Introduction

There are obviously a multitude of bacterial organisms which are infectious for the urogenital tract of humans; however, the major bacterial pathogens which specifically cause infections of the genital tract in humans include *Neisseria gonorrhoeae*, *Treponema pallidum*, and *Chlamydia trachomatis*. No animal model has been described for *N. gonorrhoeae* genital infections. Although animal models are available for *T. pallidum*, it is only acquired via the genital route, its pathogenesis being more associated with a systemic infection rather than a local genital mucosal infection. In contrast, *C. trachomatis* is restricted primarily to the genital or ocular mucosa and can thus be considered a true genital pathogen. With this in mind, this chapter focuses on animal models of *Chlamydia* which can be used to assess virulence as well as to understand the immune response.

Several animal models have been described for chlamydial genital infections of the female, including *C. trachomatis* infections of rhesus[1]

[1] D. L. Patton, C.-C. Kuo, S.-P. Wang, R. M. Brenner, M. D. Sternfeld, S. A. Morse, and R. C. Barnes, *J. Infect. Dis.* **155**, 229 (1987).

and grivet monkeys,[2] pig-tailed macaques,[3,4] marmosets,[5] and mice[6–9] as well as *C. psittaci* infections of the guinea pig,[10] cat,[11] and koala.[12]. Infections have also been characterized in male guinea pigs[13] and grivet monkeys.[14] While, without a doubt, the primate most closely approximates the human infection, it is also expensive and impractical to use for large studies. Patton has overcome this disadvantage to some degree by implanting oviduct tissue subcutaneously along the abdomen of monkeys so that multiple "pockets" are available for inoculation on a single animal.[1] While this model is extremely valuable for many types of studies, it still requires a major expense. On the other hand, the mouse and guinea pig models lend themselves well to studies involving larger numbers of animals. For this reason, the emphasis in this chapter is on these models. It should be emphasized that no one model is ideal for all studies, so that care should be taken in choosing the animal in order to address the particular research question in mind.

Infection of Guinea Pig with Agent of Guinea Pig Inclusion Conjunctivitis

Although the agent of guinea pig inclusion conjunctivitis (GPIC) is a member of the *C. psittaci*, it produces an infection in the genital tract of guinea pigs which is remarkably similar to human *C. trachomatis* genital infection with regard to pathogenesis, pathology, immunity, and ability

[2] B. R. Moller, E. A. Freundt, and P. A. Mardh, *Am. J. Obstet. Gynecol.* **138**, 990 (1980).
[3] D. L. Patton, S. A. Halbert, C. C. Kuo, S. P. Wang, and K. K. Holmes, *Fertil. Steril.* **40**, 829 (1983).
[4] P. Wolner-Hanssen, D. L. Patton, W. E. Stamm, and K. K. Holmes, *in* "*Chlamydia* Infections" (D. Oriel, G. Ridgway, J. Schachter, D. Taylor-Robinson, and M. Ward, eds.), p. 371. Cambridge Univ. Press, New York, 1986.
[5] A. P. Johnson, C. M. Hetherington, M. F. Osborn, B. J. Thomas, and D. Taylor-Robinson, *Br. J. Exp. Pathol.* **61**, 291 (1980).
[6] A. L. Barron, H. J. White, R. G. Rank, B. L. Soloff, and E. B. Moses, *J. Infect. Dis.* **143**, 63 (1981).
[7] M. Tuffrey and D. Taylor-Robinson, *FEMS Microbiol. Lett.* **12**, 111 (1981).
[8] M. Tuffrey, P. Falder, J. Gale, and D. Taylor-Robinson, *Br. J. Exp. Pathol.* **67**, 605 (1986).
[9] J. I. Ito, Jr., J. M. Lyons, and L. P. Airo-Brown, *Infect. Immun.* **58**, 2021 (1990).
[10] D. T. Mount, P. E. Bigazzi, and A. L. Barron, *Infect. Immun.* **8**, 925 (1973).
[11] J. L. Kane, R. M. Woodland, M. G. Elder, and S. Darougar, *Genitourin. Med.* **61**, 311 (1985).
[12] K. A. McColl, R. W. Martin, L. J. Gleeson, K. A. Handasyde, and A. K. Lee, *Vet. Rec.* **115**, 655 (1984).
[13] D. T. Mount, P. E. Bigazzi, and A. L. Barron, *Infect. Immun.* **5**, 921 (1972).
[14] B. R. Moller and P. A. Mardh, *Fertil. Steril.* **34**, 275 (1980).

to be transmitted sexually.[15,16] The organism is strictly an obligate intracellular parasite of superficial epithelial cells and has as its principal target tissue the exocervix.[17] Of importance is the fact that GPIC is a natural parasite of the guinea pig, so any responses studied can be assumed to be the result of natural host–parasite interactions. One problem with animal studies of human agents is that often the pathogens are not natural parasites of the animal in question, so that one has to consider an entirely different set of variables associated with host specificity. In addition, a major advantage of the guinea pig over other rodent models is that the female reproductive system is more closely related to the human than other rodents with regard to both histology and physiology. The female guinea pig has a 15- to 17-day estrous cycle with spontaneous ovulation and actively secreting corpus lutea. Thus, it can serve as an excellent model to assess hormonal effects on the infection. A variety of considerations and techniques in the use of this model will not be discussed here since they were already described in [6] on the GPIC ocular model. Nevertheless, the majority of those issues are also valid in the use of the genital tract model.

Infection and Assessment of Infection Course in Lower Genital Tract

As with ocular infections, guinea pigs from a GPIC-free colony must be used. Both male and female guinea pigs may be used in this model. However, the majority of studies have been performed with female animals because the major morbidity associated with chlamydial disease is the development of infertility resulting from salpingitis. Sexually mature guinea pigs, weighing 450–500 g, should be used for the studies.

The GPIC for infection purposes is cultured in McCoy or HeLa cells and is purified as described elsewhere in this volume (see [6]). Either a crude or gradient-purified elementary body preparation may be used. Female guinea pigs are infected intravaginally by placing the animal on its back and inserting a blunt micropipette approximately 2–3 cm into the vagina. Then 50 μl of sucrose–phosphate–glutamate (SPG) buffer (pH 7.4)[18] containing from 10^4 to 10^7 inclusion-forming units (ifu) of GPIC elementary bodies is inoculated directly into the vagina. Anesthesia is not required, but the animals should be replaced in the cage carefully in an

[15] D. L. Patton and R. G. Rank, *in* "Sexually Transmitted Diseases" (T. C. Quinn, ed.), p. 85. Raven, New York, 1992.
[16] R. G. Rank and M. M. Sanders, *Am. J. Pathol.* **140**, 927 (1992).
[17] A. L. Barron, H. J. White, R. G. Rank, and B. L. Soloff, *J. Infect. Dis.* **139**, 60 (1979).
[18] J. Schachter, *in* "Manual of Clinical Microbiology" (E. H. Lennette, ed.), p. 357. American Society for Microbiology, Washington, D.C., 1980.

attempt to prevent them from becoming excited and expelling a portion of the inoculum. A unique aspect of the guinea pig genital tract anatomy is the appearance of a vaginal membrane when the animal is not in estrus. The membrane totally prevents entry into the vagina. Therefore, when animals are to be inoculated, the membrane may be simply broken by pushing gently with the pipette tip. This procedure produces only momentary mild discomfort to the animal with no bleeding or apparent trauma.

Urethral infections in male guinea pigs may be established by extruding the penis and merely "dropping" 50 μl of inoculum onto the external meatus. An alternative method is the direct intraurethral inoculation of the same volume with a 0.76 mm (inside) by 1.22 mm (outside) diameter piece of Tygon tubing attached to a 23-gauge needle with syringe.

As in ocular infections, the course of the infection may be monitored by either the inclusion score method (see [6] in this volume) or by isolation of chlamydiae from cervical or urethral swabs. To obtain material from the female for inclusion scores, a dental stainless steel plastic filling instrument (No. D-2) is inserted about 2 cm into the vagina and the spatula end rotated against the vaginal wall. Care should be taken that epithelial cells are obtained by scraping the wall and not only by obtaining exudative material from the lumen. Most of the exudate will be inflammatory cells with few epithelial cells. The material obtained is spread onto a glass slide and stained with Giemsa after methanol fixation. The inclusion score is determined as described previously. Giemsa staining also allows examination and quantification of the exudate for inflammatory cells. Smears taken at 3-day intervals provide adequate and informative monitoring of the infection course. In general, the infection as assessed by inclusion scores reaches peak levels 6–9 days after vaginal inoculation and lasts about 18–21 days.

Urethral scrapings from male guinea pigs are obtained using the same instrument by inserting the blunt end about 0.5 cm into the urethra of the extruded penis and then scraping the urethral wall. A smear is prepared as above. Because no exudate is routinely seen in infections of male guinea pigs, less material will be apparent on the stained smear.

Cervical swabs for the isolation of GPIC are collected by inserting a Dacron swab about 3 cm into the vagina until it can go no further. The swab is then rotated against the cervix, removed, and placed into transport medium such as sucrose–phosphate buffer (2-SP) (pH 7.4).[18] Urethral swabs are collected by inserting the swab about 1 cm into the urethra, rotating, and then placing in transport medium. The isolation of chlamydiae is performed by standard procedures. Swabs may also be taken every 3 days for the mapping of the infection. Both scraping and swab procedures may be performed without anesthetizing the animal. It has been our obser-

vation that, during a primary infection, inclusion scores are sufficient to document the course of the infection, since we have never observed animals to be isolation negative in the presence of positive inclusion scores. Nevertheless, chlamydial isolation is still more sensitive, and, at the end of the infection, isolation attempts may reveal organisms for about 3 days after inclusion scores have become negative. Moreover, when assessing immunity to reinfection, it is common to find that animals are isolation positive in the absence of detectable inclusions on vaginal scrapings. Animals should be monitored until at least two consecutive negative results have been obtained to rule out sampling error.

Assessment of Infection and Disease in Upper Genital Tract of Females

Another advantage of the female guinea pig model is the inclination of GPIC to ascend the genital tract to the uterus and oviducts. Approximately 80% of uterine horns and oviducts become isolation positive for GPIC by day 7 after lower tract inoculation (Fig. 1).[16] Organisms can be isolated up to day 12 from the oviducts. The pathological response is analogous to human salpingitis with acute and chronic inflammatory components as well as plasma cell infiltration in the early stage of infection (days 5–12). Long-term (days 75–85) oviductal damage is characterized by fibrosis and chronic inflammatory response in the mesosalpinx with dilatation of the oviducts, presumably from blockage of the oviduct. Assessment of upper tract disease can readily be made by histopathological analysis of the tissues on sections stained with hematoxylin and eosin.

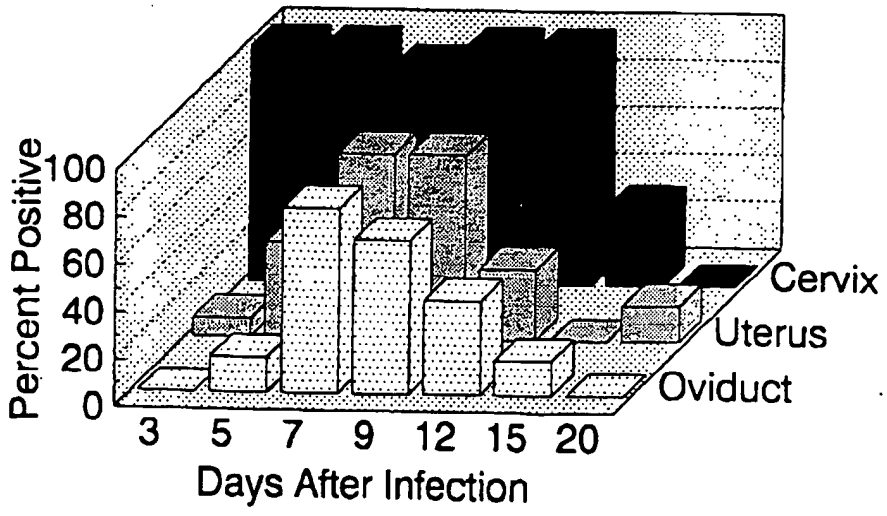

FIG. 1. Percentage of genital tract tissues positive for isolation of GPIC at varying times following intravaginal inoculation with 10^6 ifu of a chlamydial suspension.

Chlamydial inclusions and antigen can also be visualized by immunoperoxidase or immunofluorescent staining. If desired, organisms may be isolated by mincing portions of the tissue over a 60-mesh wire screen and culturing the supernatant after low-speed centrifugation to sediment tissue debris.

Assessment of histopathological reactions in the various tissues of the genital tract can be easily quantified so that data can be obtained for statistical analysis by nonparametric tests such as the Mann–Whitney *U* test. In the guinea pig, we have found that it is convenient to determine the pathological changes in the exocervix, endocervix, uterine fundus, both uterine horns, both mesosalpingeal tissues, and both oviducts. In summarizing results, one should report the data in terms of the total tissues examined, since it is variable as to whether pathological changes are unilateral or bilateral. The following parameters are assessed in each tissue: (1) acute inflammatory response (infiltration with polymorphonuclear leukocytes), (2) chronic inflammatory response (infiltration with mononuclear leukocytes including lymphocytes, monocytes, and macrophages), (3) plasma cell infiltration, (4) fibrosis, and (5) epithelial erosion (if appropriate for a given tissue). Each reaction is graded according to the following scheme.

0.5+	Trace of parameter
1+	Presence of parameter in one tissue site
2+	Presence of parameter at 1–4 foci
3+	Presence of parameter at more than 4 foci
4+	Confluent presence of parameter

Dilatation of the oviduct can also be quantitated using a similar scheme as follows:

1+	Mild dilatation of single cross section of oviduct
2+	1–3 dilated cross sections of oviduct
3+	>3 dilated cross sections of oviduct
4+	Confluent pronounced dilatation of oviduct

The data can be presented as the percentage of tissues having the parameter and/or the mean score for a given tissue.

An example of the pathological changes occurring in the mesosalpinx at varying times after infection is presented in Fig. 2. In using this system, one has a detailed descriptive and quantitative analysis of the effects of the chlamydial infection which can be used as a baseline for the study of factors associated with virulence. This is particularly important because it appears that the host response to the organism via both immunological and nonimmunological mechanisms is a significant contributor to the disease process. For instance, we have observed that repeated infection with

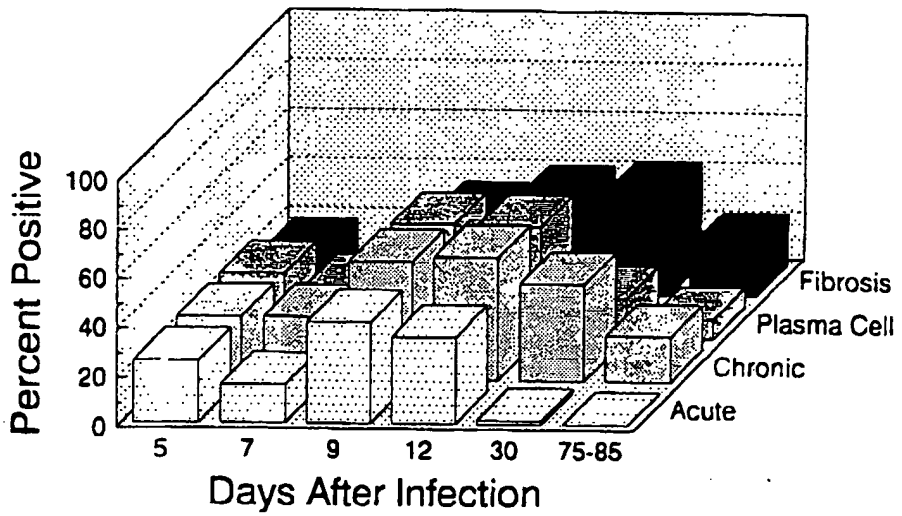

FIG. 2. Percentage of mesosalpingeal tissues positive for varying pathological changes on different days after intravaginal inoculation with 10^6 ifu of GPIC.

GPIC results in an increased percentage of guinea pigs developing tubal dilatation which is associated with infertility (R. G. Rank, unpublished data, 1993). Although the lower genital pathological response is less intense, it is more characteristic of a delayed-type hypersensitivity response than the primary infection, which has both acute and chronic inflammatory components. Moreover, chronic inflammatory and fibrotic changes in the mesosalpinx and oviduct occur and precede the development of increased tubal dilatation.

An important consideration in the evaluation of pathological changes in tissues of the female genital tract is the effect of the estrous cycle. Whereas there does not seem to be any effect on the course of genital infection as determined by inclusion scores on vaginal scrapings or on isolation of chlamydiae from cervical swabs, the production of pathological changes in the oviduct does seem to be influenced. We have noted that animals infected on day 11 of the estrous cycle (day 1 is the day of estrus) have a significantly higher percentage of oviducts with tubal dilatation and mesosalpinxes with chronic inflammation and fibrosis than animals infected on day 6 or day 16 of the cycle.[19a] Thus, in the evaluation of chlamydial disease of the upper genital tract, the stage of the estrous cycle when the animals are infected must be taken into consideration and controlled for by infecting all animals on approximately the same day of the cycle. In fact, it would be best to monitor the cycles of each animal to be used through at least 3 complete cycles to confirm the length of the

[19a] R. G. Rank, M. M. Sanders, and A. J. Kidd, *Am. J. Pathol.* **142**, 1291 (1993).

cycle for the individual animals and to verify that animals are indeed cycling. If the development of upper genital tract pathology is the desirable end point, it would then be advisable to infect all animals on day 11 of the cycle so that the highest percentage of animals develop upper tract disease.

Evaluation of Immune Response

Sera are obtained for determination of antibody by using the method of Lopez and Navia.[19] To evaluate the local antibody response in the genital tract, genital secretions can be collected by the use of a tamponlike arrangement. A 5 cm thread is tied around the center of a 2 × 10 mm preweighed section of Weck-Cel surgical sponge (Edward Weck & Co., Inc., Research Triangle Park, NC). The sponge is folded in half and is inserted into a 5 mm diameter polished glass tube, 3–4 cm in length. To collect the secretions, the guinea pig is lightly anesthetized with Innovar-vet (Pittman-Moore, Mundelein, IL, 0.08 ml/kg body weight), and the glass tube–sponge assembly is inserted approximately 2 cm into the vagina. The sponge is then pushed into the vagina with a wooden applicator stick and the glass tube removed. The thread is cut within a few millimeters of the vagina and left in place for about 1–2 hr. The sponge is removed by use of the string and, after discarding the string, is weighed again so that the weight of the secretions can be determined. The sponge may be frozen at −20° until needed. The sponge is eluted by adding 0.2 ml of phosphate-buffered saline per 0.1 g of secretion to the sponge, squeezing the sponge repeatedly to express the secretion, and finally centrifuging at high-speed in a microcentrifuge to sediment mucus and debris.

Cell-mediated immunity can be assessed in the guinea pig by the blast transformation response on peripheral blood lymphocytes as described in [6], this volume. Spleen and lymph nodes may be used as well if the animal is to be terminated. *In vivo* delayed-type hypersensitivity may also be measured by injecting 50 μl of antigen into the pinna of the ear and measuring the increase in ear thickness at 24 and 48 hr after injection. This method provides a more quantitative assessment of the response than does injection of antigen intradermally into the flank.

Infection of Mice with Agent of Mouse Pneumonitis

The agent of mouse pneumonitis (MoPn) is a biovar of *Chlamydia trachomatis*, and while its origins were most likely from the human,[20] it

[19] M. Lopez and J. M. Navia, *Lab. Anim. Sci.* **27**, 522 (1977).
[20] C. Nigg, *Science* **95**, 49 (1942).

has become adapted to the mouse and is considered to be a parasite of the mouse. Like the human oculogenital strains of *C. trachomatis*, on intravaginal infection, MoPn, remains restricted to the superficial epithelium of the cervix.[6] The major advantage of this system is related to the availability of immunological reagents for the mouse, the variety of strains of mice available including congenitally immunodeficient mice, and the relative inexpensiveness of the mouse as an experimental animal. Thus far, only studies with female mice have been performed. Studies of infection of male mice via the urethra have not been published.

Infection and Assessment of Infection Course in Lower Genital Tract

Mice may be infected intravaginally with 10^5–10^7 ifu of MoPn suspended in 2-SP or SPG buffer. Prior to inoculation, mice are injected intraperitoneally with sodium pentabarbitol (5 mg/100 g body weight). This serves to anesthetize the mice for about 1 hr so that there is less chance for loss of the inoculum. The mice are inoculated in the vagina with 30 μl of the MoPn suspension and are laid on their backs in the cage to reduce the loss into the bedding by capillary action.

A critical factor also in the mouse is the time during the estrous cycle that the animals are inoculated. We have shown that mice inoculated during estrus generally do not become infected, presumably because of the thick mucus which is commonly present. Because it would be impractical to monitor the estrous cycle in all mice, it is suggested that the mice be inoculated for 2–3 consecutive days using a fresh inoculum each day. In this way, mice will be exposed to MoPn on at least one day other than estrus. With this procedure, a high proportion of the mice become infected.

The course of infection is monitored by taking cervical swabs beginning 4–5 days after the last day of inoculation. Dacron swabs are inserted into the vagina of the mouse until they are against the cervix. The swab is then rotated with moderate pressure so that cells may be obtained and is then placed into 2-SP buffer for freezing at $-70°$. No anesthesia is required for collection of the cervical swab. The specimen is processed for isolation of MoPn in tissue culture according to standard techniques and the number of inclusion-forming units per swab determined. Obviously, the quantitation has a high variation based on the collection procedures, but the approximate inclusion-forming units as measured in a number of mice generally fall in the same range, which may be 10^4 to 10^5 at the time of peak infection.[21] The infection lasts 15–21 days, and the mice show a

[21] K. H. Ramsey, W. J. Newhall, and R. G. Rank, *Infect. Immun.* 57, 2441 (1989).

strong degree of immunity to reinfection for as long as 100–150 days after infection. Thus far, BALB/c, C57B1/6, C3H, C3H/HeJ, and outbred Swiss Webster mice have been infected in our laboratory, and no significant differences have been noted in the course of infection in any individual strain.

Interestingly, the MoPn infection of the lower genital tract appears to relatively avirulent. When congenitally athymic nude mice are inoculated, they remain infected virtually indefinitely and do not appear to suffer any ill effects.[22] We have continuously isolated MoPn from the cervix of nude mice for as long as 300 days. The organisms apparently retain their virulence because an occasional nude mouse will develop a chlamydial pneumonia, presumably as a result of aspiration of organisms during grooming. Thus, the ability of the nude mouse to remain infected provides an excellent system to assess the function of various lymphocyte populations by adoptive transfer techniques.

Infection and Assessment of Infection Course in Upper Genital Tract

The mouse–MoPn model may also be used to evaluate the effect of chlamydial infection of the oviducts on fertility and to study the basic mechanisms of salpingitis. Swenson *et al.*[23] found that infection of the oviducts could be initiated by direct injection of MoPn into the ovarian bursa. This is performed by anesthetizing the mouse with Nembutal and making a 1 cm incision on the retroperitoneal flank after clearing the area of hair with the aid of electric clippers. The ovary is immobilized, and 5 μl of an MoPn suspension (10^6 ifu/ml) in SPG buffer is injected through the fat pad directly into the ovarian bursa using a 30-gauge needle attached to a microdispensing syringe. Care should be taken as best as possible to prevent spillage so that organisms are not introduced by accident into the peritoneum. The wound may be closed simply with a wound clip without suturing the peritoneum. The procedure can be repeated for the contralateral side, or that side may be used as a control.

Generally, an acute salpingitis develops within 6–14 days. The extent of the salpingitis can be assessed grossly by observation of distention of the bursal sac, opacity due to inflammatory exudate, as well as hydrosalpinx. The tissues can also be evaluted microscopically by fixing the oviduct in formalin and staining with hematoxylin and eosin. Additionally, this model is well suited to determining the effect of the infection on fertility. Infected female mice may be housed with males beginning 5 days after

[22] R. G. Rank, L. S. F. Soderberg, and A. L. Barron, *Infect. Immun.* **48**, 847 (1985).
[23] C. E. Swenson, E. Donegan, and J. Schachter, *J. Infect. Dis.* **148**, 1101 (1983).

infection and then sacrificed 2 weeks later.[24] The animals are necropsied and the number of implants per horn of each mouse determined. One may also determine the patency of the oviduct by injecting Evans blue or methylene blue into the end of the uterine horn so that the dye may flow into the oviduct. Tubal obstruction will interfere with the ability of the dye to flow through the entire oviduct and can be equated with infertility of that particular oviduct.

Infection and Assessment of Infection Course in Mice Infected with Human Oculogenital Strains of Chlamydia

In addition to the MoPn–mouse model, mice can be infected in the genital tract with human oculogenital isolates including *Chlamydia trachomatis* serovars D, E, F, G, H, I, K, and L2.[7–9] To accomplish genital tract infection, since these agents do not have apparent biological specificity for the murine host, mice must be treated with progesterone to stabilize the epithelium in a state on anestrus. Mice are injected subcutaneously with 2.5 mg of medroxyprogesterone (Depo-Provera; Upjohn Company, Kalamazoo, MI) 10 and 3 days prior to infection. Infection is accomplished by inoculating via the vaginal route with 10^7–10^8 ifu of the chlamydiae-containing suspension. Depending on the serovar, organisms can be isolated from the cervix 7–8 weeks after infection. In general, the recovery of organisms by isolation is lower than with MoPn at comparable times. Salpingitis can also be studied with the human oculogenital serovars by using the same methodology as described for MoPn, except that progesterone should be given to facilitate infection.

[24] C. E. Swenson and J. Schachter, *Sex. Transm. Dis.* **11**, 64 (1984).

[8] Animal Models for Meningitis

By MARTIN G. TÄUBER and ANDRÉ ZWAHLEN

Introduction

Approximately 15,000 cases of bacterial meningitis occur each year in the United States, while other parts of the world have a substantially higher incidence. Even when treated with highly active antibiotics, the disease is fatal in 5–40% of the patients and causes neurologic sequelae

EXHIBIT A3

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Correlation of infertility with altered tubal morphology and function in mice with salpingitis induced by a human genital-tract isolate of *Chlamydia trachomatis*

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Summary. Progesterone-treated C3H mice were inoculated into the uterus or ovarian bursa with a human genital tract isolate of *C. trachomatis* (serovar E), or with control medium alone. The mice were then observed at various times up to 260 days after inoculation. Before being killed the mice were given pituitary gonadotrophins to induce ovulation. Eggs were sought in the oviducts and ciliary activity in the fimbrial and ampullary sections of the oviducts was determined by light microscopy, before detailed examination by scanning electron microscopy.

Eggs were visible in all control oviducts and both mucosal ultrastructure and ciliary activity appeared normal. By contrast, eggs were not recovered from the inoculated oviducts of mice infected intrabursally, nor was ciliary activity observed up to 28 days after inoculation. After this, ciliary activity reappeared but eggs were still not transported to the oviduct. Ultrastructural studies suggested that severe mucus congestion accompanied by tubal oedema and loss of ciliated epithelia play a major role in the aetiology of chlamydial-induced tubal damage. Infertility following chlamydial salpingitis could be associated with failure of egg transportation to the oviduct. Egg transport was still impaired even when luminal ciliary activity, ultrastructural integrity and patency had recovered.

Our results suggest that chlamydial salpingitis in this mouse model closely resembles the human disease in its pathology and consequences for fertility, making the model particularly relevant for research on chlamydial vaccine development.

Keywords: Chlamydia trachomatis; salpingitis; animal models

Introduction

Salpingitis caused by *Chlamydia trachomatis* in women often leads to infertility or a marked increased risk of ectopic pregnancy (Chow *et al.*, 1987; Robertson & Ward, 1988). In many regions of the world it is likely that *C. trachomatis* is the single major cause of tubal infertility. Animal models are essential to study the immunopathological basis of *Chlamydia*-induced tubal damage and its prevention. Non-human primate models are of particular relevance as they appear to resemble closely the human disease (Patton, 1985). Unfortunately, non-human primates are scarce, expensive and thus inappropriate for the large scale breeding experiments necessary to ascertain the consequences of chlamydial infection for subsequent fertility. The upper genital tract of mice can be infected with the *C. trachomatis* mouse pneumonitis agent and the mice may subsequently develop infertility (Swenson *et al.*, 1983; Swenson & Schachter, 1984), although this murine strain of *C.*

trachomatis differs from human isolates both antigenically and in DNA re-association studies. Consistent chlamydial infection of the mouse oviduct can also be achieved using human genital tract isolates of *C. trachomatis* (Tuffrey *et al.*, 1986a, b). This model, for the first time, permitted long-term, statistically adequate studies on defective egg transport and the mechanisms responsible for *Chlamydia*-induced tubal infertility. Egg transport was impaired even in the absence of tubal occlusion. One possibility is that the epithelial surface of the oviduct is damaged, impairing its functional integrity. There have been several studies on the ultrastructure of the oviduct following chlamydial infection (reviewed by Patton, 1985), but there has been no attempt to correlate sequential ultrastructural changes at the luminal surface of the oviduct with functional markers of tubal integrity such as ciliary activity, ovum transport and fertility. This paper reports the consequences of chlamydial infection for mouse oviduct function and ultrastructure, in relation to our previous studies on *Chlamydia*-induced infertility in the mouse.

Materials and Methods

Mice. Syngeneic C3H mice, 6–8 weeks old, bred in the specific pathogen-free (SPF) unit at the National Institute for Medical Research (Mill Hill, London, UK) were used.

C. trachomatis *strains.* *C. trachomatis* NI.1 was isolated from the endocervix of a contact of a man with non-gonococcal urethritis and was serotyped as serovar E, one of the commonest genital tract serovars. The isolate was passed serially in McCoy cells. Chlamydiae from the tenth passage, suspended in 2SP sucrose-phosphate buffer (68·46 g sucrose; 2·088 g K_2HPO_4; 1·088 g KH_2PO_4; water 1 litre; pH 7·2) containing 10% fetal calf serum, were used for subsequent inoculation. This inoculum was free of contaminating mycoplasma or bacteria and was known to cause salpingitis in mice.

Infection of mice. Progesterone treatment enhances murine infection of mice with *C. trachomatis* (Tuffrey & Taylor-Robinson, 1981; Tuffrey *et al.*, 1986a). All the mice were therefore given a single dose of 2·5 mg progestagen (Depo-Provera, Upjohn, Kalamazoo, MI, USA) subcutaneously 7 days before inoculation with strain NI.1 (test) or 2SP suspending medium (control). The animals were anaesthetized with 0·1 ml/30 g body weight of a mixture made up with 1 part Hypnorm® (Janssen Pharmaceutical Ltd, Grove, Oxford, UK) in 2 parts water and 1 part Hypnovel® (Roche Products, Welwyn Garden City, Herts, UK). The inoculum was introduced either directly into the right uterine horn (intrauterine) or under the right ovarian bursa (intrabursal) and thus into an oviduct. Mice were given 5×10^3 inclusion-forming units (ifu) of strain NI.1 by the intrauterine route or 5×10^4 ifu by the intrabursal route. The surgical procedures have been described previously (Tuffrey *et al.*, 1986a).

Detection of chlamydiae. Cervical/vaginal swabs were taken on Days 7 and 13 after inoculation to confirm infection of the genital tract. Each swab was expressed in 1 ml of cold 2SP medium which was stored in liquid nitrogen until subsequent inoculation by centrifugation onto cycloheximide-treated McCoy cells for conventional chlamydial isolation (Thomas *et al.*, 1977).

Detection of eggs. Administration of pituitary gonadotrophins to female mice induces ovulation, irrespective of their oestrous phase. Infected mice and their controls were induced to ovulate from Day 8 to Day 260 after inoculation by giving them i.p. injections of 5 i.u. PMSG (Folligon: Intervet, Cambridge, UK) intraperitoneally, followed by 5 i.u. hCG (Chorulon: Intervet) about 45 h later. Eggs were sought in the oviducts on the following day. The mice were killed and ovaries and oviducts were dissected out and placed in a watch glass in 0·2–0·3 ml 0·5 M-Hepes-buffered MEM medium. Clumps of eggs surrounded by cumulus cells could be seen in control oviducts at this time by using a stereomicroscope.

Assessment of ciliary activity. After the oviducts had been examined for the presence or absence of eggs, the ciliated fimbrial and ampullary sections of the tubes were dissected out under a stereomicroscope. The ampullary region was then sliced along its length with a microsurgical knife to expose the luminal epithelial surface. The specimen was examined with an inverted microscope to determine the presence and vigour of actively beating cilia within both regions, then transferred to fixative for scanning electron microscopy (SEM).

SEM studies. Mouse oviducts were fixed in 5% (v/v) glutaraldehyde in 0·1 M-cacodylate (pH 7·4) and stored at 4°C in fixative until processing. After a wash in buffer (0·1 M-cacodylate and 0·5 M-sucrose pH 7·4) the ducts were post-fixed for 1 h in 2% (w/v) osmium tetroxide, rinsed in distilled water, dehydrated through graded ethanols, then critical-point dried in liquid carbon dioxide using a Polaron E3000 drier. In some cases oviducts were first straightened out and attached to pieces of card before fixation, then were cut transversely with a small scalpel into 6 or 7 segments before processing. The dried segments were mounted with the luminal surfaces uppermost and coated with approximately 20 nm gold/palladium to facilitate examination in an Hitachi S800 field emission SEM (resolution 2 nm) at 15 kV accelerating voltage.

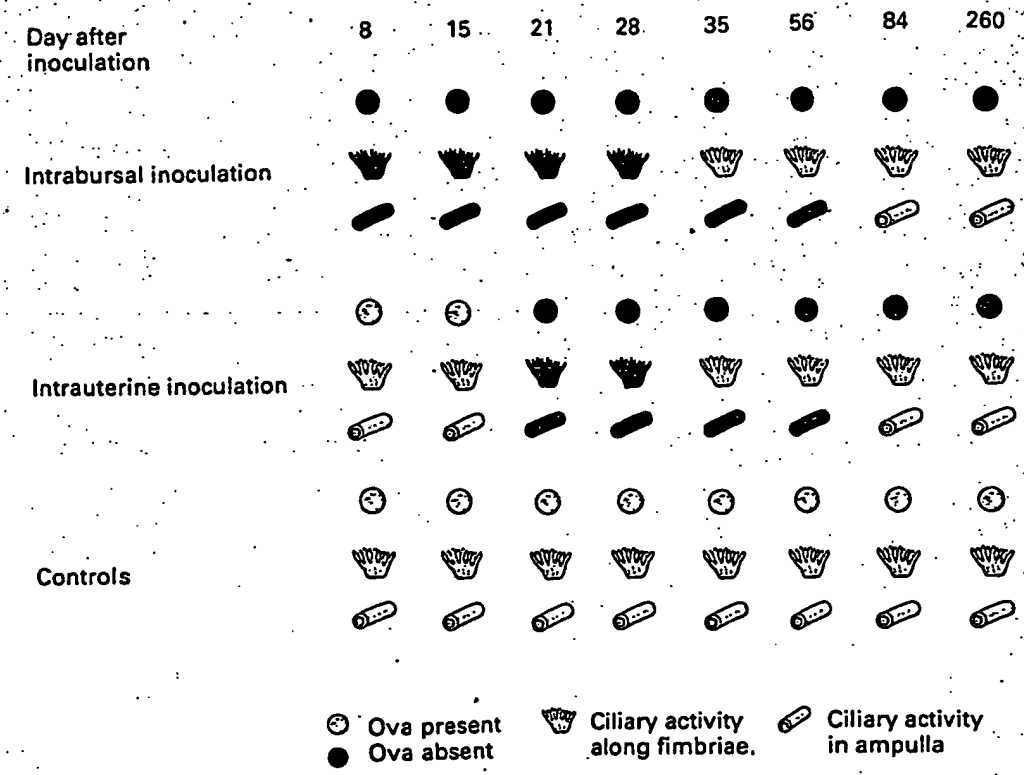

Fig. 1. Summary diagram showing the effects of intrabursal or intrauterine inoculation of *Chlamydia trachomatis* on ovum transport and ciliary activity in mouse oviducts. Normal or impaired function is indicated by the shaded or unshaded symbols respectively.

Results

The ultrastructure study used 30 mice: 15 were inoculated intrabursally (10 with NI.1 and 5 with 2SP) and the remaining 15 were inoculated via the intrauterine route. One mouse from each group plus a control mouse was killed at various times from Days 8 to 260 after inoculation. Data on oviduct function and ciliary activity were available for 24 of these mice as shown in Fig. 1.

Confirmation of infection

Chlamydiae were recovered from vaginal swabs taken from all mice inoculated into the uterus with NI.1 on both Days 7 and 13 after inoculation, and from all except 2 mice inoculated intrabursally on Day 13 after challenge. However, there was evidence at necropsy for both of these last 2 mice of hydrosalpinx formation.

Egg transport

Eggs were found in both oviducts of all the control mice which were inoculated with Medium 2SP alone (Fig. 1). By contrast, no eggs were transported to the oviducts on the injected side in any of the mice inoculated with chlamydiae via the intrabursal route, nor were they found in oviducts on the non-injected side (data not shown) in these mice killed on Days 28, 35 and 42 after inoculation. The failure of egg transport occurred later in mice inoculated by the intrauterine route. Egg transport was impaired after Day 15 on the injected side or from Day 35 on the non-injected side and no eggs were recovered from oviducts on either side in mice killed 42 days after inoculation.

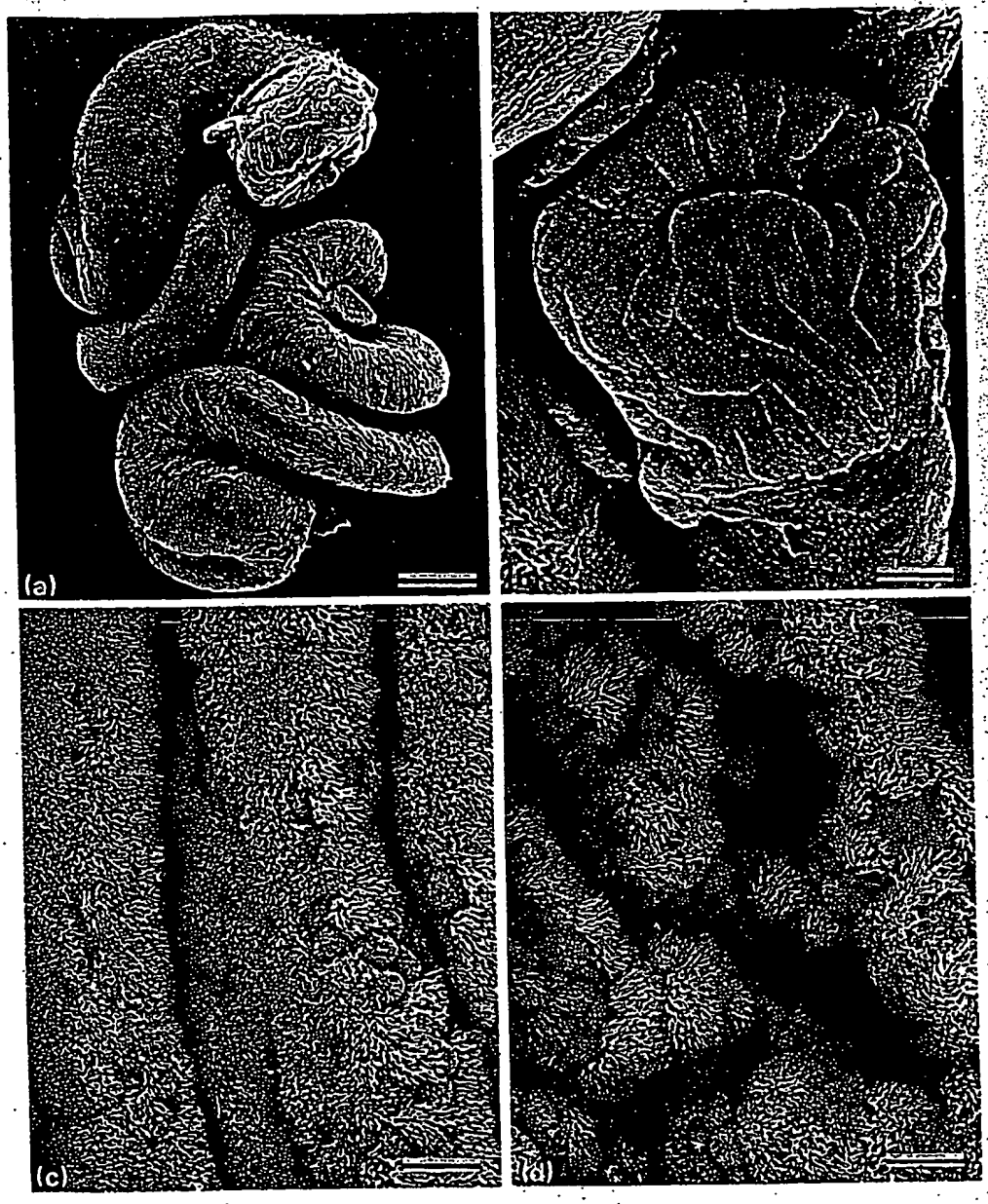

Fig. 2. Scanning electron micrograph (SEM) of normal oviducts from control mice showing (a) whole oviduct, (b) the infundibulum, (c) ciliated epithelia from the infundibular region, (d) ciliated and non-ciliated epithelia of the ampullary region. The bars represent (a) 218, (b) 84, (c) 12 and (d) 9 μm.

Ciliary activity

Loss of ciliary movement in the fimbrial and ampullary regions of the oviduct was associated with failure of egg transportation up to Day 28 after inoculation. In oviducts studied on Days 35, 42 and 56 after inoculation, some fimbrial ciliary activity had returned, although the eggs were not transported and there was still no apparent ciliary activity within the ampullary region. From

Fig. 3. SEM of the mucosal surface of the mouse oviduct 8 days after intrabursal inoculation with *C. trachomatis*. The cilia are already disorganized and there are numerous globules of dried mucus. The bar represents 7·5 μm.

Day 84 after inoculation, both fimbrial and ampullary ciliary activity appeared normal but egg transport was still impaired.

SEM studies

The typical appearance and size of normal mouse oviduct is shown in Fig. 2. The normal lining of the infundibular region was fimbriated (Fig. 2b, c) with a high density of ciliated epithelia (Fig. 2c). In the ampullary region (Fig. 2d) the ciliated epithelial cells were less dense and interspersed with columnar epithelial cells which were covered with microvilli.

At 8 days after intrabursal inoculation, the infected tube failed to transport ova and its walls appeared thickened and oedematous. The surface of this tube was characterized by marked mucus hypersecretion (Fig. 3) with matted and apparently disorganized cilia but few inflammatory cells. Mucus hypersecretion in intrabursally inoculated oviducts reached a peak at Day 15, with the ampullary region of the tube blocked with exudate (Fig. 4a). This exudate consisted mainly of mucus droplets (Figs 4b, c) with the occasional inflammatory cell (Fig. 4c). The underlying epithelium was disorganized, with a loss of ciliated epithelial cells and breakdown of ciliary structure (Fig. 4d). Mucus hypersecretion continued to be observed as long as 84 days after infection. Secondary bacterial infection, probably gram-negative rods, was occasionally observed in tubes with mucus hypersecretion (Fig. 5a, b). Marked disorganization of the ciliated epithelial surface was still apparent at Day 35.

Hydrosalpinx formation was first observed as early as 28 days after intrabursal infection. Figure 6(a) shows a transversely sectioned normal (control) tube. Fimbriae are apparent within the

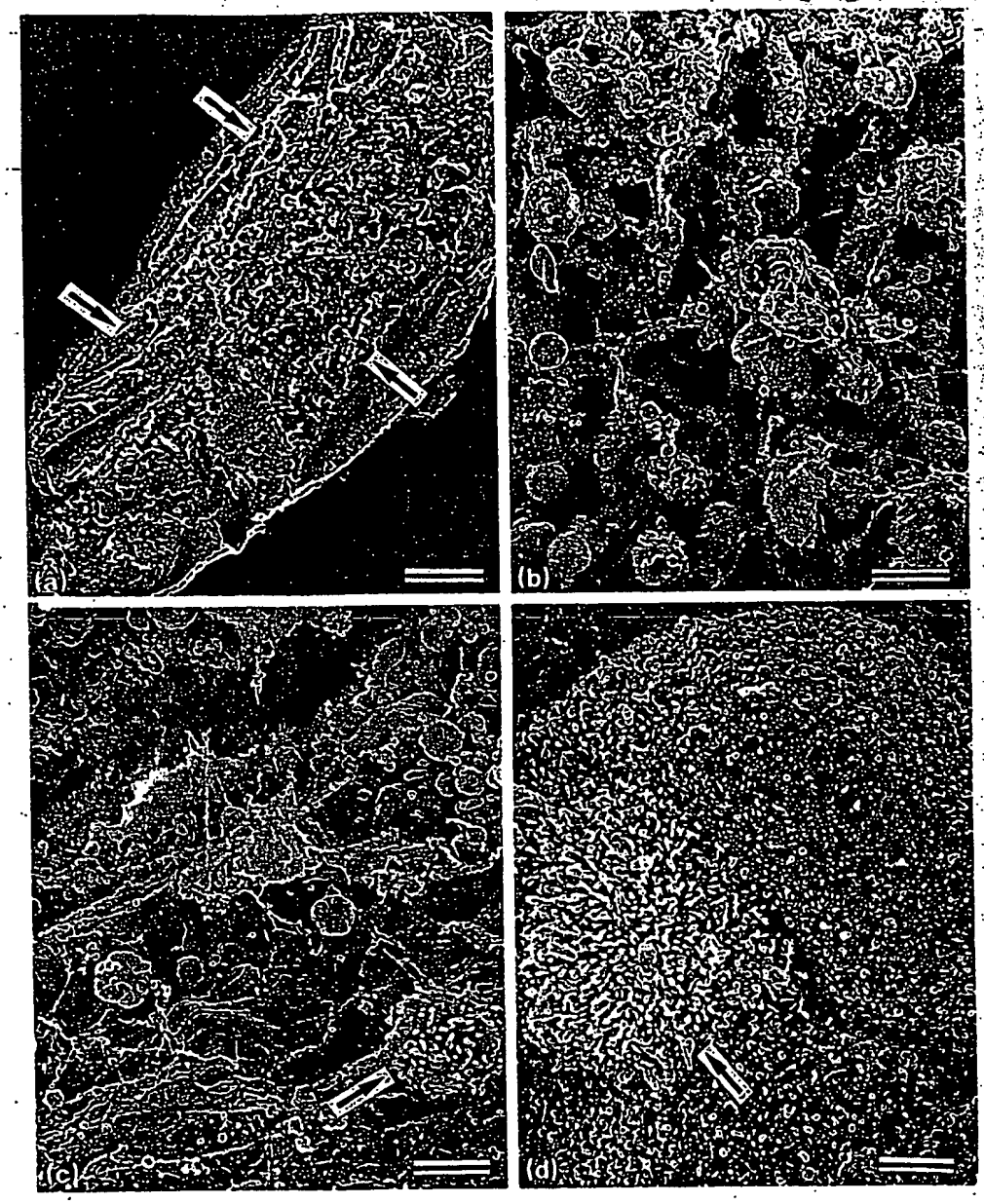

Fig. 4. SEM of mouse oviduct 15 days after intrabursal inoculation. (a) Longitudinal section along the oviduct showing the oviduct lumen (arrows) already blocked with exudate. (b) Higher power of (a): the exudate consists mainly of dried mucous secretion plus cell debris. (c) Occasional inflammatory cells (arrowed) were interspersed amongst this debris. (d) A small area of the epithelium showing the major extent of epithelial disruption and destruction of the cilia (arrowed) beneath the exudate. The bars represent (a) 104, (b) 8, (c) 3 and (d) 2·5 μm.

unobstructed lumen of this tube. By contrast Fig. 6(b) shows the exudate-obstructed lumen of a similar region of a *Chlamydia*-infected tube. The marked hydrosalpinx and epithelial compression frequently associated with such a blockage is shown in Fig. 6(c). Within this compressed region the mucosal lining of the tube nevertheless showed evidence of patchy regeneration of the ciliated

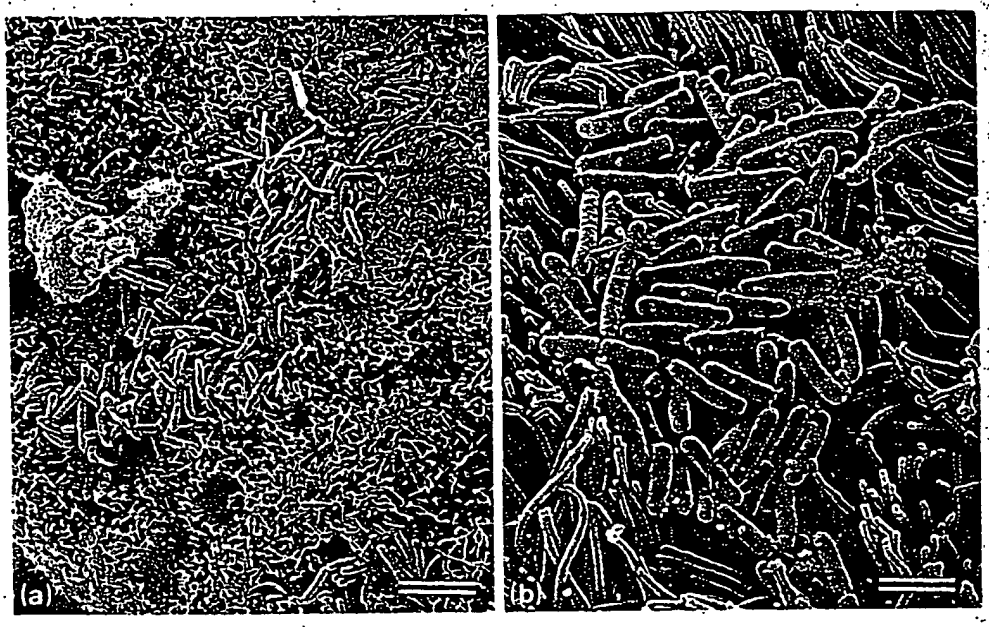

Fig. 5. SEM of mouse oviduct 56 days after intrauterine inoculation. (a) Low-power picture showing the extent of secondary infection with bacterial rods. (b) Higher magnification view of similar area. The bars represent (a) 4 and (b) 1·5 μm.

epithelium (Fig. 6d). Occasional inflammatory cells were observed on the epithelium (Fig. 7a), readily distinguishable by their size and topography from mucus droplets but the regenerated cilia were still matted together, presumably with mucus (Fig. 7b). Blockage of the ampullary region of the tubes and associated hydrosalpinx was noted as late as 71 days after infection.

By 161 and 260 days after intrabursal challenge, ovum transport was still impaired but paradoxically the oviduct lumen looked relatively normal, with just patchy areas of epithelial disruption and moderate amounts of mucus secretion (data not shown).

Discussion

The major advantage of the mouse model is that it is cheap, animals are readily available, and it is possible to correlate pathological changes in the oviduct with their consequences for fertility. Alternative, non-primate, experimental models of chlamydial salpingitis exist, but in our view are less satisfactory. For example, in the rabbit model (Patton *et al.*, 1982) there is doubt as to whether long-lasting infection is ever established. Both the guinea-pig model (White *et al.*, 1979) and the cat model (Kane *et al.*, 1985) use *C. psittaci* rather than the natural human pathogen *C. trachomatis* as the infecting organism. An alternative mouse model (Swenson *et al.*, 1983) uses a non-human isolate of *C. trachomatis*, the mouse pneumonitis agent.

The use of progesterone to enhance chlamydial infection of the genital epithelia in the present model might be criticized. The function of the progesterone, a natural steroid, is believed to be the stabilization of the genital epithelia which otherwise vary with the oestrous cycle, increasing the number of target cells available for chlamydial infection (Tuffrey & Taylor-Robinson, 1981). Infection can be achieved in the absence of the steroid (Tuffrey *et al.*, 1986a), but it is clear that progesterone in no way diminishes the inflammatory response to chlamydial infection in the mouse.

The results of the present study showed that impairment of ovum transport occurred as early as 8 days after intrabursal inoculation of *C. trachomatis* and had spread to the uninoculated

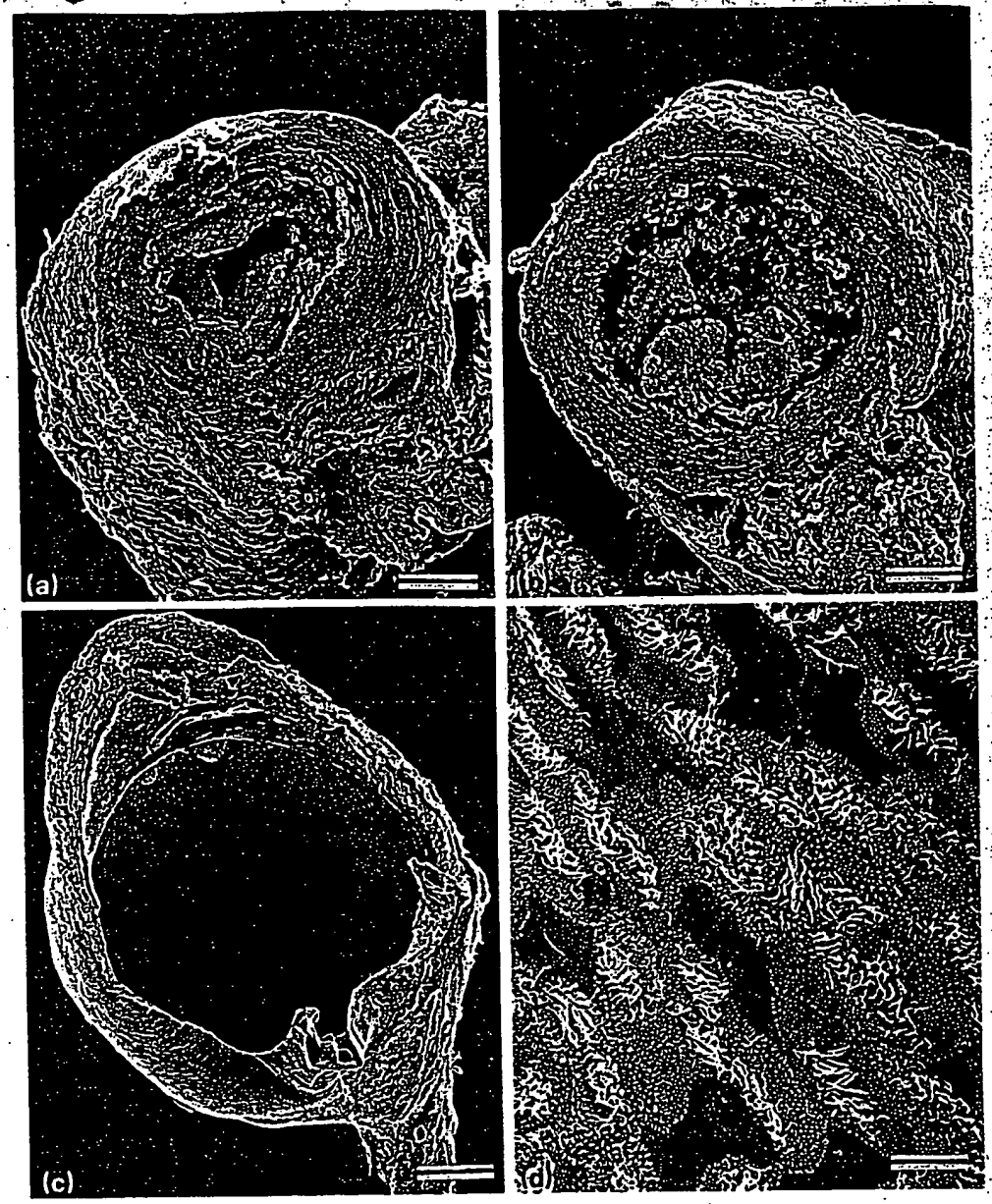

Fig. 6. SEM of transverse section of normal and *Chlamydia*-infected mouse oviduct. (a) Ampullary region of normal oviduct from a control mouse showing an unobstructed lumen with associated intraluminal fimbriae. (b) Similar region of infected oviduct 57 days after inoculation. The lumen is occluded with mucus and cell debris. (c) Infected oviduct with greatly enlarged lumen due to tubal obstruction and hydrosalpinx. (d) Detail within the lumen of (c) showing patchy regeneration of the compressed epithelial lining. The bars represent (a) 52, (b) 42, (c) 111 and (d) 11 μm.

contralateral tube by 28 days after infection. We assume this is the result of canalicular spread of chlamydiae via the uterus, as previously reported (Swenson *et al.*, 1983; Tuffrey *et al.*, 1986a). Intrauterine inoculation of *C. trachomatis* delayed the onset of impaired ovum transport to 21

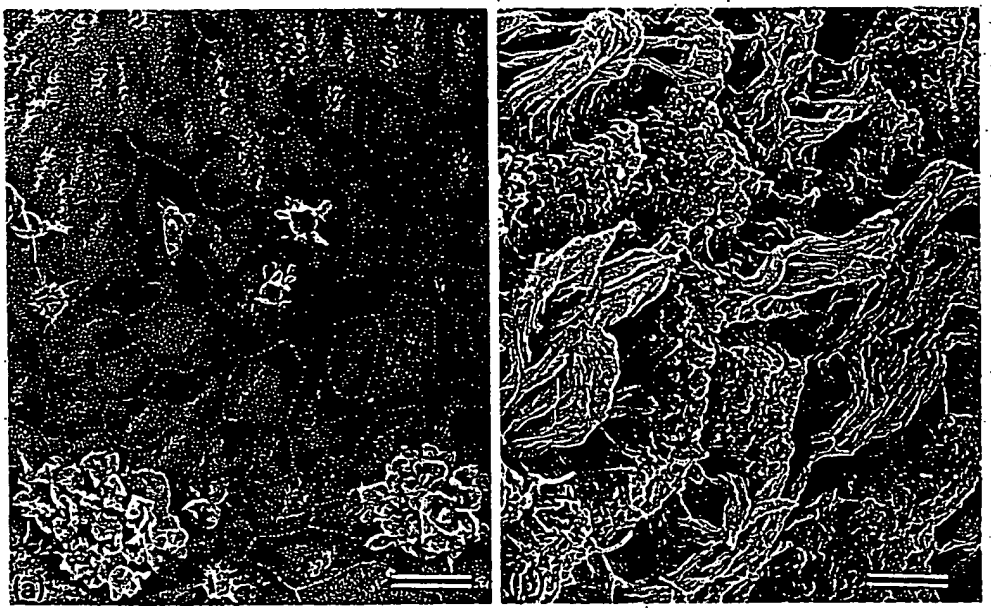

Fig. 7. SEM of inoculated mouse oviduct 57 days after challenge. (a) Inflammatory cells on the epithelial surface of a tube with a hydrosalpinx. (b) Cilia matted together with mucous secretion. The bars represent (a) 23 and (b) 3 μm.

days. However, once egg transport was impaired the effect was long-lasting, with mice 260 days after challenge still affected irrespective of the inoculation route. This impairment of egg transport following superovulation correlated well with infertility as previously reported (Tuffrey *et al.*, 1986b). Pathological changes observed in this study by SEM also correlated well with those previously observed by light microscopy (Tuffrey *et al.*, 1986a).

The most striking pathological change, first observed 8 days after intrabursal inoculation of *C. trachomatis*, was mucus congestion accompanied by tubal oedema and the loss of ciliated epithelial cells from the oviduct lumen. Few inflammatory cells were observed on the oviduct mucosa, although such cells have been observed in paraffin-wax sections (Tuffrey *et al.*, 1986a). It is likely that by Day 8 any initial acute inflammatory response in the lumen has already subsided. A similar loss of ciliated epithelial cells and an absence of inflammatory cells was observed in pig-tailed macaques (Patton *et al.*, 1987), together with evidence of T-cell infiltration in the stroma. It is unclear whether mucus congestion is primarily the result of impaired ciliary clearance induced by the loss of ciliated epithelial cells or is due to hypersecretion in response to the infection or associated lymphokines. The question is important as this study suggests that mucus accumulation is a major factor leading to tubal blockage. Moreover mucus in abnormal amounts may have been the "amorphous" substance observed at the luminal surface of blocked human Fallopian tubes (Tam *et al.*, 1988). Perhaps mucus interferes with ovum transport in a manner similar to polycations, preventing the formation of transient adhesive bonds between the tip of the cilium and elements of the cumulus mass (Norwood *et al.*, 1978). One possibility which could be tested in the mouse model is whether mucolytic or anti-inflammatory therapy early in salpingitis might reduce subsequent tubal damage.

Blockage of the narrow, infundibular region of the oviduct with mucus, interspersed inflammatory cells and necrotic epithelia was maximal 15 days after intrabursal challenge and was similar to that previously observed in paraffin-wax sections (Tuffrey *et al.*, 1986a). In a number of mucus-congested and *Chlamydia*-infected tubes we observed secondary infection with bacterial rods. We speculate that, once mucus congestion with impaired ciliary clearance becomes established, the

affected oviduct becomes vulnerable to secondary bacterial infection arising from the cervix. Mucus congestion, loss of ciliated epithelia, secondary bacterial infection or hydrosalpinx were only observed in *Chlamydia*-infected animals and never in control animals inoculated with the suspending medium alone. After intrabursal inoculation in those tubes not affected by hydrosalpinx, mucus congestion began to decrease gradually and ciliary activity to increase around Day 35. Occasional inflammatory cells were also present on the luminal surface at this time. It is possible that phagocytic cells, e.g. macrophages, play a role in eliminating the mucus.

Hydrosalpinx is a common outcome of chlamydial infection in both mice and women once the tube is blocked and drainage prevented. In the mouse model, hydrosalpinx formation was first observed 28 days after challenge, occurred in 10 animals, and was initially associated with marked compression and destruction of the underlying epithelium as has been reported for occluded human Fallopian tubes (Fedele *et al.*, 1984; Tam *et al.*, 1988). Recovery of the epithelium and ciliary activity in the mouse occurred later in tubes with a hydrosalpinx. Recovery of ciliary activity with time would explain the apparent disagreement in SEM studies of blocked human Fallopian tubes as to whether there is extensive loss of ciliated epithelium (Patek & Nilsson, 1977; Fedele *et al.*, 1984; Tam *et al.*, 1988).

Oviducts collected 160–260 days after chlamydial infection had an apparently normal epithelial surface. Ciliary activity was vigorous and there were only patchy signs of epithelial disorganization. Nevertheless, ovum transport remained impaired in these late specimens. We can only speculate on the cause. One possibility is that the tubal lumen may still have been blocked at its narrowest region most difficult to examine by SEM. However, in women with tubal occlusion, surgical restoration of tubal patency often fails to achieve fertility and there is a high frequency of subsequent tubal ectopic pregnancy (Zamberletti *et al.*, 1983; Tam *et al.*, 1988). Chronically blocked human Fallopian tubes with hydrosalpinx show little correlation between the extent of de-ciliation and subsequent fertility (Fedele *et al.*, 1984). Clearly factors other than tubal occlusion or deciliation must also be involved. Perhaps the metachronal rhythm of the regenerated cilia is disorganized and so ovum transport is impaired. Further investigations using both transmission electron microscopy and time lapse cine-microphotography would be necessary to investigate these possibilities.

The mouse model of chlamydial salpingitis resembles the human disease in the infecting chlamydial strains, the canalicular spread of infection, its pathology and the consequences for fertility. Preliminary immunological studies with this model indicate the importance of both host and *Chlamydia*-related factors for immunity (Tuffrey *et al.*, 1982, 1986a). It seems highly unlikely that the severe consequences of chlamydial infection for human fertility can be prevented by community health programmes alone in the absence of an effective vaccine. Given the major advances now being made in chlamydial immunochemistry, including the successful expression of recombinant chlamydial antigens (Pickett *et al.*, 1988) and the identification of neutralizing epitopes on the major outer membrane protein of *Chlamydia* (Zhang *et al.*, 1987; Conlan *et al.*, 1988), together with a greatly increased understanding of mucosal immunity, we believe it is time to assess the value of potential vaccines for preventing *Chlamydia*-induced infertility. We anticipate that this mouse model will play a major and relevant role in such a programme.

We thank Dr David Taylor-Robinson, Division of Sexually Transmitted Diseases, MRC Clinical Research Centre, for encouraging this collaborative study. Part of this work was supported by a grant from the Human Reproduction Programme of the World Health Organization.

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Vice President, Research and Development (2003 to 2004)

Vice President, Research (2000 to 2003).

Director, Research (1997 to 2000).

Director, Molecular Biology (1995 to 1997).

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Senior Research Molecular Biologist (1990 to 1994).

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Philosophy Doctorate (High Honors)
September 1978 to September 1984
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Bachelor of Arts (Summa Cum Laude)
September 1973 to June 1978
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Microbiology / Biochemistry (Majors)
University of Tennessee, Knoxville, TN 37919.

Vice President, Research

- **Executive Management and Administrative Responsibilities:**

  Reports directly to CEO
  Responsible for all R&D operations
  Executive Committee Member
  Responsible for developing company's IP portfolio
  Functioned as Company's Chief Technical Officer
  Financing / M&A presentations & diligence / Technology acquisitions

- **R&D Responsibilities:**

  Recruited, organized and directed an R&D staff of ~40 scientists into two operating divisions **AntexBiologics** and **AntexPharma.** Responsible for developing and executing company's Biologics and Pharma R&D plans having an annual combined budget of ~$12M.

AntexBiologics:

The mission of AntexBiologics is to prevent or treat select gastrointestinal and sexually transmitted infections and/or their related sequale using vaccine technologies. Program activities range from antigen discovery, biochemical / epidemiological characterization, preclinical immunogenicity and efficacy studies and initial human clinical trials (Phase I/II). Based on clinical outcome, vaccine candidates will be evaluated in later stage clinical studies (Phase III) or out-licensed. AntexBiologics consists of

Microbiology, Molecular Biology, Protein Chemistry, Immunology, and Fermentation groups.

Vaccine Products / Projects Under Development:

TRACVAX - Recombinant *C. trachomatis* subunit vaccine => Status: Phase I

TWARVAX – Recombinant *C. penumoniae* subunit vaccine => Status: Preclinical

GONOVAX - Recombinant *N. gonorrohea* subunit vaccine => Status: Preclinical

HELIVAX – Inactivated *H. pylori* whole cell vaccine => Status: Phase II

HELIVAX II – Augmented *H. pylori* whole cell vaccine => Status: Preclinical

ACTIVAX – Inactivated *S. sonnei* whole cell vaccine => Status: Phase I

Mucosal Adjuvant Development => Discovery / Preclinical

Vice President, Research

AntexPharma:

The mission of AntexPharma is to identify new chemical entities (NCEs) having both broad and narrow spectrum antibacterial activity. Program activities encompass identification/selection of potential antibacterial core structures, SAR-based analogue syntheses, NAACLS-based MIC and MBC analyses, synthetic scale-up, preliminary formulation and in vivo efficacy / bioavailability studies. A lead candidate suitable for topical application will be through initial stage human clinical trials (Phase I/II). Based on clinical outcome, lead candidates will be evaluated in later stage clinical studies (Phase III) or out-licensed. The Pharma unit consists of Antibacterial Research and Organic Synthesis groups.

Anti-infective Products / Projects Under Development:

Heterocyclic alkanes – AP158 => Status: Preclinical / Phase I

Fused lactams => Status: Discovery / Preclinical

Derivativized quinolones => Status: Discovery / Preclinical

Novel Antibacterial Drug Targets => Status: Discovery

- **Development Experience:**

***Helicobacter pylori* – HELIVAX / *Campylobacter jejuni* Vaccines:**

Organized the development of defined, bovine component-free media for use in 300l-scale fermentation of *C. jejuni* and *H. pylori* whole cell vaccines. Directed development of a purification process for the clinical manufacture of a recombinant *H. pylori* subunit vaccine (HP30).

***Chlamydia trachomatis* – TRACVAX:**

Directed the development of a purification process for the clinical manufacture of a recombinant *C. trachomatis* subunit vaccine antigen (CT110) and a formulation procedure for mucosal delivery of the CT110 protein.

- **Manufacturing / Clinical Trial Experience:**

***Chlamydia trachomatis* – TRACVAX**

Supervised cGMP manufacture (seed banks, fermentation, purification, formulation, fill, release testing) of high value *C. trachomatis* antigen and an ADP-ribosylating mucosal adjuvant for use in Phase I trials.

Designed Phase I safety and immunogenicity trial for the evaluation of the lead antigen candidate. Directed the preparation of TRACVAX Phase I Safety and Immunogenicity IND, Investigator's Brochure and responses to FDA comments. Identified appropriate CROs and clinical trial sites.

***Helicobacter pylori* – HELIVAX**

Supervised cGMP manufacture (seed banks, fermentation, inactivation, fill, release testing) of a chemically inactivated *H. pylori* whole cell antigen. Participated in the design of Phase I/II dose-ranging and immunogenicity trials.

Director, Research

- **Management & Administrative Responsibilities:**

Responsible for technical / budgetary management of Smith-Kline Beecham–MicroCarb Human Vaccine R&D discovery and preclinical evaluation programs including:

Project/target identification, project planning (definition of critical path technical milestones/goals); program budget formulation and tracking; project timeline preparation and monitoring; technical resource allocation, IP strategy and staging.

- **R&D Responsibilities:**

***Helicobacter pylori* / *Campylobacter jejuni* / *Shigella spp.* (*S. sonnei, S. flexneri*)**

Development and scale-up of growth conditions to be used to produce virulence-enhanced; whole cell vaccines, preclinical animal model immunogenicity and efficacy evaluation of inactivated whole cell (+/-) mucosal adjuvant vaccines; Phase I & Phase II Clinical Trial design in collaboration with Naval Medical Research Institute (NMRI, Enteric Disease Program) and SKB.

Vice President, Research

Chlamydia trachomatis / Chlamydia pneumoniae / Neisseria gonorrohea

Identification of subunit vaccine candidate(s) using adhesin-receptor technology (ART); biochemical, epidemiological and immunologic characterization of subunit candidates; production of recombinant proteins in *E. coli* and/or baculovirus; purification / formulation of recombinant antigen for preclinical evaluation; and preclinical animal efficacy evaluation.

Moraxella catarrhalis / Neisseria meningitidis* B / *Haemophilus influenzae

Computational analysis of public/proprietary genomic databases to identify ORFs encoding putative adhesins, hemolysins, type III secretion factors, porins, lipoproteins, pili, redox enzymes (catalase, SOD, PLA), and iron / heme sequestration proteins; production of recombinant proteins in *E. coli* and/or baculovirus; purification / formulation of recombinant antigen for preclinical evaluation; and preclinical animal efficacy evaluation.

Director, Molecular Biology:

- **R&D Responsibilities:**

Responsible for all of company's Molecular Biology activities including:

Genomic database analyses, ORF identification, oligonucleotide design and synthesis, PCR and recombinational cloning, expression (primarily *E.coli*, baculovirus, yeast, CHO), DNA sequencing, genomic library construction, Southern / Northern analyses, knock-out mutant construction, site-directed mutagenesis, subtractive hybridizations, expression vector construction, solubility / activity analysis of expressed protein(s) and recombinant product verification.

In Silico Genomics – SmithKline Beecham Biologics / MCHV

Responsible for cloning/expression and purification of >40 ORFs from select respiratory pathogen genomic databases for in vitro / in vivo evaluation as vaccine candidates:

Director, Molecular Biology:

PCR cloning and expression of membrane-associated ORFs in *E.coli* / Pichia pastoris, RFLP variability analysis of homologue / orthologue ORFs from geographically distinct clinical isolates, DNA sequence analysis of RFLP-variable ORFs, PCR-based genomic walking and sequencing to complete partial ORFs originating from "high linkage" number databases, ^{3}H-glycerol / ^{3}H-palmitic acid labelling (+/-) globomycin to confirm lipidation of recombinant proteins, construction of novel expression vectors to achieve N-terminal lipidation of recombinant proteins.

VeroTest™

Directed final development of RELISA kit for detecting verotoxin (Shiga-like toxins) producing strains of *E. coli* (e.g.O157:H7), performance / stability testing, GMP manufacture and field evaluations of Research Use Only test.

- **Administrative Responsibilities:**

  Patent Liaison:

  Assist patent counsel (Pennie & Edmonds) in prosecuting company's U.S. and foreign patent portfolio. Prepared technical responses to Office Actions; represented company at PTO examiner interviews; oversaw/coordinated preparation of patent applications and invention disclosures. Results: 3 issued U.S. patents, 2 Notice of Allowance, 5 new applications filed, and 2 pending invention disclosures.

  Radiation Safety Officer:

  State RAM license preparation, renewal, and amendments; reviewed and approved protocols involving use of radioactive materials; authorize procurement, receipt, inventory, use and disposal of all licensed materials; coordinate timely health physics monitoring activities (personal and environmental0; decommissioning of RAM use areas.

  Chairman, Safety Committee:

  Organized company's biological, chemical, and radiological safety program; reviewed safety policies and defined mission, oversee safety training requirements

Senior Research Molecular Biologist:

- **R&D Responsibilities:**

  Animal Therapeutics – Bovine Embryo Cloning:

  Cloned bovine parathyroid hormone-like protein (bPTHlp) from both cDNA and genomic libraries. Expressed and purified recombinant bPTHlp from *E. coli* and stably transfected methotrexate-amplified CHO-K1 cells. Evaluated bPTHlp for use as a therapeutic treatment for parturient hypocalcemia in cattle via a UMR108 osteosarcoma cAMP-based bioassay.

  Identified and employed PCR-based bovine genomic VNTR (dinucleotide) markers for defining origin and genetic parentage of chimeric embryos and fetal tissues during *in utero* development as part of ABS embryo cloning program.

  Designed, directed synthesis, purification and evaluated amphipathic synthetic peptides in a fluorescent liposome assay for in vitro cell-cell fusigenic activities. Designed amphipathic synthetic peptides and evaluated via a fluorescent liposome assay for use as an in vitro oocyte-blastomere cell-cell fusigenic agent.

- **Administrative Responsibilities:**

  Evaluate External Technologies:

  Appraise external intellectual property (technical presentations, scientific notebooks, research reports, patents and patent applications) for "in-house" value and communicate findings and recommendations to senior management, business developers, and patent attorneys.

Research Molecular Biologist:

- **R&D Responsibilities:**

  Specialty Chemicals – Artificial Organ Development:

  Cloned and expressed a *Vibrio proteolyticus* metalloprotease in *E. coli* and *Bacillus subtilis* as a readily available supply of enzyme for use in bioreactor synthesis of artificial sweeteners. Performed site-directed mutagenesis to increasing thermal stability of a novel *Vibrio* protease to generate enzyme variants having elevated temperature stability profiles.

Research Molecular Biologist:

Initiated development of PCR-based diagnostic assays for detecting and identifying the primary fungal and bacterial agents which cause peritonitis in CAPD patients

Implemented a Western blot system to identify and quantify major serum proteins (albumin, transferrin, immunoglobins, etc.) to define the in vivo adherence and exclusion properties of polysulfone hollow fibers destined for use in an artificial hybrid pancreas.

In collaboration with ANU researchers, developed a PCR-based embryo sexing assay for use in bovine cloning program.

Grantsmanship:

NIH / NIAID – R43 AI3661-01.
Small Business Innovative Research (SBIR) Grant, Phase I.
"Adhesin Vaccine for *Chlamydia trachomatis*"
Funded September 1994 – February 1995 **$74,565**
Principal Investigator

DoD / Office of Naval Research – N00014-96-C-0362.
Small Business Innovative Research (SBIR) Grant, Phase I.
"Rapid Test for Diagnostics of Campylobacter Enteritis and Shigella Dysentery in Operational Ship and Field Environments"
Funded July 1996 – January 1997 **$76,820**

Principal Investigator

NIH / NIAID – R44 AI36617-02.
Small Business Innovative Research (SBIR) Grant, Phase II.
"Development of an Adhesin Vaccine for *Chlamydia trachomatis*"
Funded September 1996 – September 1998 **$838,992**
Principal Investigator

DoD / US Army Medical Research Acquisition Activity – DAMD17-01-C-0026.
Small Business Innovative Research (SBIR) Grant, Phase I.
"Development of a Microbead Whole Cell Delivery System for Oral Immunization Against traveler's Diarrhea."
Funded January 2001 – June 2001 **$68,314**
Principal Investigator

MIPS (Maryland Industrial Partnership Program) – Contract Agreement #2609
University of Maryland, School of Pharmacy
"Airway Mucous Secretion Inhibitors"
Funded August 2000 – July 2001 **$101,685**
Contract Manager

MIPS (Maryland Industrial Partnership Program) – Contract Agreement #2609.28
University of Maryland, School of Pharmacy
"Airway Mucous Secretion Inhibitors / Phase II"
Funded August 2001 – July 2002 **$184,603**
Contract Manager

NIH / NIAID – AI56452-02.
"Novel Adjuvants for Biodefense Vaccines"
Funded September 2003 – August 2005 **$523,116**
Co-Principal Investigator

Issued Patents:

USPTO 6,642,023. Chlamydia protein, gene sequence and uses thereof.
Inventors: Jackson; W. James and Pace; John L.; Assignee: Antex Biologics, Inc.

USPTO 6,693186. Neisseria meningitidis protein, nucleic acid sequence and uses thereof.
Inventors: Jackson; W. James and Harris; Andrea M.; Assignee: Antex Biologics, Inc.

USPTO 6,756,493. Neisseria species protein, nucleic acid sequence and uses thereof.
Inventors: Jackson; W. James and Harris; Andrea M.; Assignee: Antex Biologics, Inc.

USPTO 6,887,843. Chlamydia trachomatis proteins and uses thereof.
Inventors: Jackson; W. James and Pace; John L.; Assignee: Antex Biologics, Inc.

Published Patent Applications:

"Cloning and Expression of the Bovine Parathyroid Hormone Related Protein and Use as a Prophylactic Treatment for Parturient Paresis in Dairy Cattle." Applicant: W.R. Grace & Co.-Conn.; Inventor: Washington James Jackson: PTO Application Number: 860,708.

"Neisseria meningitidis Polypeptide Gene Sequences and Uses Thereof." Applicant: Antex Biologics Inc.; Inventors: W. James Jackson and Andrea M. Harris; US 09/388,089.

"Neisseria spp. Polypeptide Gene Sequences and Uses Thereof." Applicant: Antex Biologics Inc.; Inventors: W. James Jackson and Andrea M. Harris; US 09/388,090.

"Chlamydia spp. Polypeptide, Gene Sequences and Uses Thereof": Applicant: Antex Biologics Inc.; Inventors: W. James Jackson and John L. Pace; US 08/866,592.

"Chlamydia spp. Polypeptides, Gene Sequences and Uses Thereof": Applicant: Antex Biologics Inc.; Inventors: W. James Jackson; US 09/459,032.

"Helicobacter pylori Polypeptide, Gene Sequences and Uses Thereof": Applicant: Antex Biologics Inc.; Inventors: Jing-Hui Tian, W. James Jackson and Richard I. Walker; US 08/639,936.

"Novel 2-Aryloxyphenol Derivatives as Antibacterial Agents"; Applicant: Antex Biologics Inc.; Inventors: Liren Huang, Wei-Tong Wang, Alenka G. Tomazic, Joanna M. Clancy and W. James Jackson; US 09/388,090.

"4-Subsituted 2-Aryloxyphenol Derivatives as Antibacterial Agents" Applicant: Antex Pharma Inc.; Inventors: Liren Huang Alenka G. Tomazic, Joanna M. Clancy and W. James Jackson; US 11/214,772.

2-(2 Or 4-Subsituted Aryloxy)-Phenol Derivatives as Antibacterial Agents": Applicant: Antex Pharma Inc.; Inventors: Liren Huang Alenka G. Tomazic, Joanna M. Clancy and W. James Jackson; US 11/289,362.

Novel 2-Heterooryloxy-Phenol Derivatives as Antibacterial Agents": Applicant: Antex Pharma Inc.; Inventors: Liren Huang Alenka G. Tomazic, Joanna M. Clancy and W. James Jackson; US 11/320,755.

Publications:

1. Jackson, W.J. and A. O. Summers. 1982. Polypeptides Encoded by the Mercury-Resistance (mer) Operon. J.Bacteriol. 149:479-487.

2. Jackson, W. J. and A. O. Summers. 1982. Biochemical Characterization of the HgCl2-Inducible Polypeptides Encoded by the mer Operon of Plasmid R100. J.Bacteriol. 151:962-970.

3. Barrineau, P., P. Gilbert, W. J. Jackson, C. Slater-Jones, A. O. Summers, and S. Wisdom. 1985. The DNA Sequence of the Mercury-Resistance Operon of the IncFII Plasmid NR1. J.Mol.Appl.Gen. 2:601-619.

4. Barrineau, P., P. Gilbert, W. J. Jackson, C. Slater-Jones, A. O. Summers, and S. Wisdom. 1985. The Structure of the mer Operon, p. 701-718. In D. R. Helinski and S. N. Cohen (eds.), Plasmids in: Bacteria. Plenum Press, New York.

5. Jackson, W. J. Jackson, R. C. Prince, and B. L. Marrs. 1986 Energetic and Topographic Properties of a B875 Light-Harvesting Mutant of Rhodopseudomonas capsulata. Biochem. 25:8440-8446.

6. Prince, R. C. and W. J. Jackson. 1986. The Role of the Light-Harvesting I Antenna Proteins in the Correct Insertion of the Photochemical Reaction Center of Rhodobacter capsulatus and Rhodobacter sphaeroides. Progress in Photosynthesis Research (Biggins, J., ed.) Martinus Ni jhoff, Dordrecht 4:721-724.

7. Jackson, W. J. and R. C. Prince. 1986. Genetic and Sequence Analysis of a Rhodobacter capsulatus Mutant Unable to Properly Insert the Photochemical Reaction center into the Photosynthetic membrane.
Progress in Photosynthesis Research (Biggins, J., ed.) Martinus Nijhoff, Dordrecht 4:725-728.

8. Heltzel, A. G., W. J. Jackson, D. A. Gambill, and A. O. Summers. 1987. Overexpression and DNA-Binding Properties of the mer-Encoded Regulatory Protein from Plasmid NR1. (Tn21). J.Bacteriol. 169:3379-3384.

9. Jackson, W. J. , P. T. Kiley, S. Kaplan, and R. C. Prince. 1897. On the Role of the Light-Harvesting B880 Apparatus in the Correct Insertion of the Reaction Center of Rhodobacter capsulatus and Rhodobacter sphaeroides. FEBS.Lett. 215:171-174.

10. Sesno, J.A., R. M. Overton, and W. J. Jackson. 1994. Genomic and cDNA Cloning of the Bovine Parathyroid Hormone-Related Protein (bPLP). Manuscript submitted Gene.

11. Stice, S. L., N. S. Strelchenko, W. J. Jackson, C. A. Keefer, and L. W. Matthews. 1994. Bovine Embryonic Stem Cell Lines: Demonstration of Pluripotency Following Nuclear Transfer. Manuscript submitted, Theriogenology.

12. McKenzie, R. L., R. I. Walker, G. S. Nabors, C. Carpenter, G. Gomes, E. Forbes, J. H. Tian,, H. H. Yang, J. L. Pace, W. J. Jackson, and A. L. Bourgeois. 2004. Safety and Immunogenicity of an Oral, Inactivated, Whole-Cell Vaccine for Shigella sonnei: Preclinical Studies and a Phase I Trial.

Abstracts:

1. Jackson, W. J. and A. O. Summers. 1979. Proteins Synthesized in minicells harboring cloned mer DNA. Abstracts of the Third Annual Mid-Atlantic Regional Extrachromosomal Elements Meeting. Plasmid. 3:235-242.

2. Jackson, W. J. and A. O. Summers. 1980. Genetic and polypeptide analysis of the mer operon. Abstracts of the Annual Meeting of the American Society for Microbiology, H-35, p.341.

3. Jackson, W. J., F. A. Bohlander, and A. O. Summers. 1981. The mer operon: Polypeptides and a promoter. Annual Meeting of the Genetics Society of America, June 1981.

4. Jackson, W. J. and A. O. Summers. 1982. The relation of the HgCl2-inducible R-factor membrane proteins to HgCl1-resistance in Escherichia coli. Abstracts of the Annual Meeting of the Society for
Microbiology, CCI-113, p.526.

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R. Keefe, G. Nabors, J. Tian, R. Walker, Y. Feng, R. Harris, J. Jackson; Antex Biologics, Gaithersburg, MD.

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H. Lu, A. M. Harris, G. S. Nabors, W. J. Jackson;
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22. The Immunogenicity and Protective Capacity of BioThrax® Are Significantly Enhanced by CPG 7909 M. GU[1], J. CLANCY[1], P. HINE[1], C. BOTEZAN[1], L. SIMON[1], B. KINTNER[1], L. BONDOC[1], I. SIM[2], C. NIELSEN[3], G. NABORS[1], W.J. JACKSON[1], L. GIRI[1]
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